[GANN 52, 109~119; June, 1961]

# HISTOLOGICAL STUDIES OF GASTRIC MUCOSAL CANCER WITH SPECIAL REFERENCE TO RELATIONSHIP OF HISTOLOGICAL PICTURES BETWEEN THE MUCOSAL CANCER AND THE CANCER-BEARING GASTRIC MUCOSA

**(Plates V~VII)**

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The higher incidence of gastric cancers in this country, compared with Europe, America and other countries, has been statistically proven by many investigators.[26),39),40),46)] In Japan, more than sixty thousand people die of this disease every year. Many reasons, to which can be ascribed the high incidence of gastric cancer, have been proposed by many investigators from various fields of study, but from the standpoint of pathological histology, the following deserves attention as having been most neglected in the studies so far. Namely, over 90 per cent of the cases, which are diagnosed clinically as gastric cancer, are progressive ones and are hardly available for the study of histogenesis of gastric cancer. But recently, cases of early gastric cancer, which might be expected not to recur over five years after the surgical resection, have increased gradually, owing to the improvement of diagnostic methods. In order to clarify the histological features of the early gastri cancer andc thus to contribute somewhat to the prevention and early detection of gastric cancer, the following investigations were undertaken based on these early cases.

## Materials and Methods

Forty two cases of gastric mucosal cancers, which were obtained from 2865 cases of the gastrectomy conducted during past 10 years at the Yokoyama's Hospital in Nagoya City, were used in this study.

All of the resected stomachs received careful histological examinations in our laboratory and the advanced cancers and the early cancer, in which the cancerous lesions occupied mainly the mucosa but slightly invaded into the submucosa, were excluded from this study. The gastrectomy material consisted of 1869 cases of gastric or duodenal ulcer, 900 cases of gastric cancer, 13 cases of non-epithelial gastric tumors such as myoma, neurinoma, hemangioma and sarcoma, etc., 11 cases of gastric adenomatous polyp and 72 cases of others, in which no remarkable changes were recognized.

The resected stomachs were cut along the greater curvature and the entire

mucosa, after being photographed, were immediately fixed in 10% formalin solution. After fixation of the stomachs, more than ten tissue blocks prepared from all areas including the diseased parts, were embedded in paraffin, and sections were then stained with hematoxylin and eosin.

## RESULTS

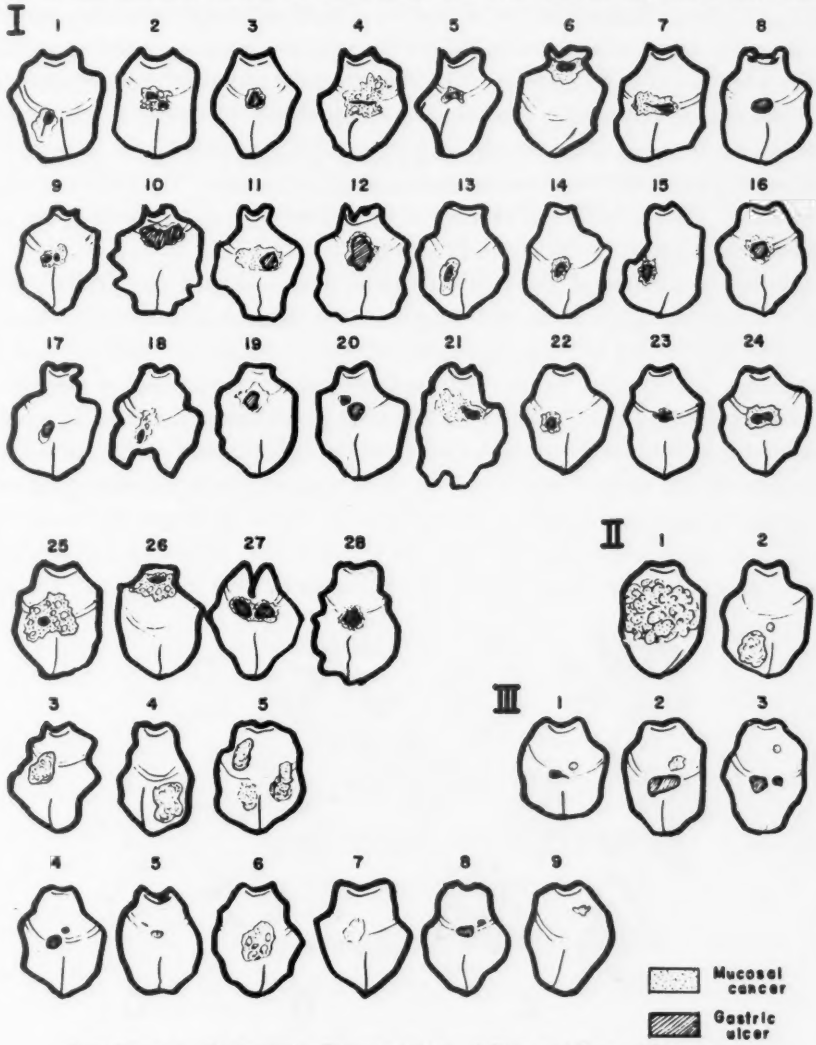
Table 1. Age and sex of patients, and histological types, location, etc., of mucosal cancer examined in this study.

| Group | No. | Age | Sex | Mucosal cancer | | | | | Complication |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Location | Size (cm.) | Histolog. types A | B | C | |
| I | 1 | 41 | m | c, p. | | + | + | | |
| | 2 | 25 | m | py-c, l. | | | + | + | |
| | 3 | 41 | m | py-c, l. | | + | | | |
| | 4 | 45 | m | py-c, l. | | + | + | | |
| | 5 | 40 | m | py-c, l. | | | + | + | |
| | 6 | 37 | f | py, l. | | | + | + | |
| | 7 | 45 | m | py-c, l. | | | | + | |
| | 8 | 47 | m | py-c, l. | | + | | | |
| | 9 | 33 | f | py-c, l. | | | + | + | |
| | 10 | 55 | m | py-c, l. | | | + | + | |
| | 11 | 45 | m | py-c, a. | | | + | + | |
| | 12 | 41 | m | py, l. | | | + | | |
| | 13 | 40 | f | c, p. | | | + | + | |
| | 14 | 33 | m | py-c, l. | | | | + | |
| | 15 | 60 | m | py-c, p. | | | + | | |
| | 16 | 69 | m | py-c, l. | | + | + | | |
| | 17 | 68 | m | py-c, l. | | + | + | | |
| | 18 | 48 | f | c, l. | | | + | + | |
| | 19 | 68 | m | py-c, l. | | + | | | |
| | 20 | 59 | m | py-c, p. | | + | | | |
| | 21 | 49 | f | py-c, l. | | | | + | |
| | 22 | 58 | f | py-c, p. | | + | | | |
| | 23 | 45 | m | py-c, l. | | + | | | |
| | 24 | 63 | m | py-c, l. | | + | | | |
| | 25 | 51 | f | py-c, l. | | | | + | |
| | 26 | 62 | f | py. l. | | | + | + | |
| | 27 | 49 | f | py-c, l. | | | + | + | |
| | 28 | 43 | m | py-c, l. | | | + | + | |
| II | 1 | 65 | f | py, | (diffuse) | + | | | |
| | 2 | 44 | m | c, p. | 7.0×7.0×5.0 | + | | | |
| | | | | c, l. | 1.0×1.0×0.8 | | | | |
| | 3 | 49 | f | c, p. | 5.0×4.0×2.5 | + | | | |
| | 4 | 50 | m | c, a. | 5.0×4.0×3.0 | + | | | |
| | 5 | 42 | f | py, p. | 5.0×3.0×2.0 | + | | | |
| | | | | c, p. | 3.0×3.0×0.5 | + | | | |
| | | | | c, a. | 1.5×1.5×0.5 | | | | |
| III | 1 | 45 | m | py-c, a. | 3.3×0.2 | + | | | Ulcer |
| | 2 | 52 | m | py, a. | 0.2×0.1 | + | | | 〃 |
| | 3 | 58 | m | py, a. | 0.3×0.2 | + | | | 〃 |
| | 4 | 71 | m | py-c, l. | 0.3×0.2 | + | | | 〃 |
| | 5 | 61 | m | py-c, l. | 0.5×0.2 | + | + | | 〃 |
| | 6 | 51 | f | py-c, l. | 6.5×4.5 | | + | + | (−) |
| | 7 | 55 | m | py-c, p. | 2.5×2.0 | + | | | (−) |
| | 8 | 52 | m | py-c, a. | 0.3×0.2 | + | + | | 〃 |
| | 9 | 46 | m | py, a. | 0.5×0.3 | + | | | (−) |

py: pylorus l: lesser curvature a: anterior wall p: posterior wall

Among 900 cases of the gastric cancer, only 42 cases (4.7%) were the mucosal cancer, in which the cancerous lesions were limited to the gastric mucosa alone. Histological examinations revealed that these mucosal cancers consisted of 28 cases of ulcer-cancer (Group I), 5 cases of polyp cancer (Group II) and 9 cases of superficial spreading erosive cancer without ulcer or polyp (Group III).

Site of the cancers varied in each case, but over half of the cases, the cancer

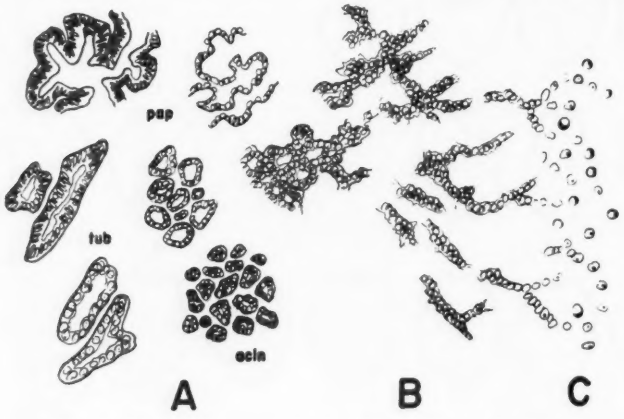



Text-figure 1. Diagramatic Representation of Site, and Size of Mucosal Cancers Examined in This Study. (Groups I, II and III)

were situated in the intermediate zone between the corpic and the pyloric gland areas of the lesser curvature. In Group II, however, predisposition of site to the cancer was not recognized. (Table 1, Text Fig. 1)

In gross appearances, size of the cancers of Group I varied from minute one, which was hardly detectable in naked-eye examination, to fairly extended one, in which the cancerous tissues occupied the mucosa surrounding the ulcers accompanied by ill-defined erosion. Rugged margin of the ulcer was not always found in this group. In Group II, intramucosal cancer showed more or less polypoid elevations and easily diagnosible by naked-eye examinations. The cancers of multicentric origin were found in three out of five cases. Size of the eroded cancer in Group 3 was small in general but in a few cases the cancer occupied fairly extended area, noncancerous mucosa within these lesions remaining like islands in the sea. Thickening or induration of the gastric walls and changes of the serosa were never found in the cases without complication of the ulcers (Text Fig. 1).

Histological pictures of the mucosal cancer were classified into following three types, according to the grade of differentiation of the cancerous epithelia (Text-Fig. 2). The first type (A) takes appearent tubular or glandular structure and was subdivided into tubular, acinar and papillary forms. In the second type (B), the cancerous epithelia show little or no glandular arrangement but show an irregular cord-like or trabecular structures without forming any glandular cavity. Occasionally this type of the cancers was named by us as reticular cancer, because of the appearance of the cancerous epithelia forming network. In the third type (C), cancer cells showed no epithelial masses and dissociated cancer cells infiltrated diffusely within the mucosa. Usually these cells contained mucus in their cytoplasm. The above



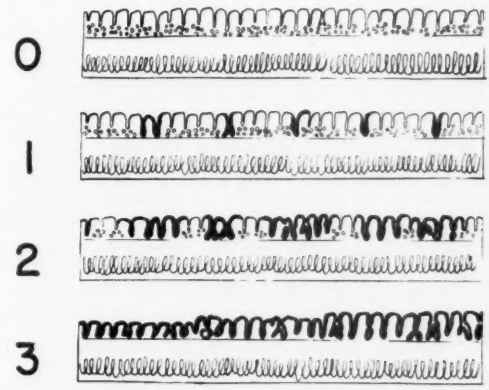

Text-figure 2. Diagrams showing Three Histological Types (A, B and C) of Mucosal Cancer.

classification is based on the histological picture presented in one microscopical field and in fact, in many cases, coexistence of A and B types or B and C types and furthermore transitional picture from A type to B, or B type to C were not infrequently observed. When these pictures were observed in a single case, this was classified into mixed type.

Histological types of the mucosal cancer were arranged according to age of the patients. All cases in the second and third decade belonged to B—C type or C type, while most of those over fifth decade were classified into A type or A-B type. Those in the fourth decade have not shown such an intimate histological relation and they were classified into various types (Table 2).

Table 2. Relationship between Histological Types of Gastric Mucosal Carcinoma and Age of Patients.

| Age of patients \ Histological types | A | A–B | B | B–C | C | |
|---|---|---|---|---|---|---|
| 70 yrs. | | | | | | 1 |
| 60 | | | | | | 8 |
| 50 | | | | | | 9 |
| 40 | | | | | | 20 |
| 30 | | | | | | 3 |
| | | | | | | 1 |
| | 17 | 6 | 3 | 12 | 4 | 42 |




Text-figure 3. Diagrams showing Four Grades (0, 1, 2 and 3) of Intestinal Metaplasia in Non-Cancerous Pyloric Mucosa.

The histological types of the mucosal cancer were then compared with the degree of intestinal metaplasia of the non-cancerous pyloric mucosa, which was classified into four grades according to the distribution of the metaplastic epithelia (Text-Fig. 3. Thick line indicate metaplastic epithelia). Cases with no or slight metaplasia (I.M. 0–0.5) belonged always to B to C type, but those with high grade of metaplasia (I.M. 2.0–3.0) belonged almost all to A to B type. Such a histological inclination was

Table 3. Relationship between Histological Types of Gastric Mucosal Carcinoma and Grades of Intestinal Metaplasia.

| Histological types / Grade of I. M | A | A–B | B | B–C | C | |
|---|---|---|---|---|---|---|
| 3.0 | □□ ○ ××× | × | | | | 7 |
| 2.5 | □□ □ | □□ | | | | 4 |
| 2.0 | ○ ××× ○ × | □ × | □ | □ | | 9 |
| 1.5 | □ ○ | □ | | □□□□□ × | □ | 9 |
| 1.0 | □ ○ | | □ | □□ | □□ | 7 |
| 0.5 | | | | □□ | | 2 |
| 0 | | | □ | □□ | □ | 4 |
| | 17 | 6 | 3 | 12 | 4 | 42 |

I.M.: Intestinal Metaplasia □ Ulcer Cancer ○ Polyp Cancer × Superficial-spreading erosive Cancer

not observed in cases with moderate metaplasia (Table 3).

From histogenetical standpoints, all of the polyp cancer presented a histological picture of A type or A–B type and showed a papillary or papillo-tubular form. All of superficial spreading erosive cancer had a picture of A or A–B type with exception of one case. Ulcer-cancer, however, showed no such histological inclination. But attention must be called to the fact that the cancer having a ptcture of B to C types were almost all included in this type (Tables 2 and 3).

Mode of growth within the mucosa is generally classified into extensive and infiltrative forms. The former occurs by gradual extension of the cancerous tissues toward the surrounding mucosa and has a sharp border-line between these two areas. Gradual replacement of non-cancerous epithelia by cancerous one is characteristic of the course taken by A type cancer. The infiltrative growth differs from the extensive

one entirely and is manifested by diffuse and infiltrative growth of isolated mucus-filled cancer cells into the mucosal stroma. This type of growth is most clearly seen in C type cancer.

Lymphnode metastasis was scarcely seen in the mucosal cancer. But a point deserving attention is the presence of metastasis in few cases such as Nos. 21 and 26 of Group 1. In these cases there were already found small clumps of cancer cells in peripheral sinuses of the regional lymph nodes.

## Discussion

Mucosal cancer of the stomach was first reported by Versé[51] in 1909. He examined 12 cases of mucosal cancers, which were preceded by ulcer or polyp, out of 10000 cases of autopsical materials. Konjetzny[16] and Bertrand, then, described another type of mucosal cancer, in which no ulcer or polyp were present, and former named this type of cancer as "Oberflächliche Schleimhautkrebs". Following these reports, Cabot[6] (1935), Ewing[9] (superficial erosive cancer, 1936). Mallory[18] (cancer in situ, 1946), Stout[43] (superficial spreading carcinoma, 1942), Rössle[37] (Oberflächenkrebs, 1940), Ayabe[1] (mucosal carcinoma, 1949), Morisaki[20] (mucosal cancer, 1954), Torium[48] (superficial spreading cancer, 1955) and Bocian[2] (carcinoma in situ, 1958) reported similar case or cases. Recently, Nagayo[31] and Takagi[45] reported a detailed and statistical studies on the mucosal cancer of the stomach.

Variability of histological pictures of advanced gastric cancer is frequently reported and discussed by many investigators[3),7),8),34),38)44),47),50] and is, in fact, experienced in our routine histological examinations. For reasons of this variability, influences of many extrinsic factors such as maturity of the cancer tissues, effects of degenerative or necrotic products caused by circulatory disturbances of the cancer, mixed infection due to ulceration of the cancer, corrosive effects of acid gastric juices, changes of histological environments due to heterologous growth of the cancer and decrease of host resistance, etc; must be taken into consideration. In mucosal cancer, however, such extrinsic factors seemed to be almost negligible. From this point of view, it is reasonable to consider that the gastric mucosal cancer has a relatively essential or basic histological pictures. Variations in histological pictures of the mucosal cancer, therefore, can be assumed to be due to the factors intrinsic in the cancerous tissues themselves.

Hebbel[11], Morson[21),22)] and Oota[36] investigated a relationship between histological features of advanced gastric cancer and that of the cancer-bearing gastric mucosa, but no pertinent data have been obtained, because of difficulty of determining whether the changes which occurred in the mucosa are primary or secondary. In this respects, too, the gastric mucosal cancer presents a suitable materials for this field of study. It is repeatedly reported that intestinal metaplasia is originated

from a faulty regeneration of the gastric mucosa[10),17),33),35)] and is seen occasionally on the mucosa surrounding the cancerous lesion. Therefore, complete denial of possibility that the metaplasia is caused secondarily by invasive growth of the cancer cannot be made, but this type of metaplasia is easily distinguishable from that of primary one owing to the distribution of the metaplastic lesions.

As has been pointed out by many investigators[5),12),15),43)], intestinal metaplasia of gastric mucosa are rare and slight in the young but with advance in age, its frequency and grade increase gradually. The same results were obtained by our examination[31)], made on about 1456 cases of the resected stomachs. Many pathologists[19),24),33),46),47)] pointed out that the frequency of differentiated adenocarcinoma is much greater in older people than in the younger and histological characteristic of the latter[32)] group is a high incidence of mucous carcinoma composed of disseminated signet-ring cells. The above two results support the view that the histological pictures of the gastric cancer are mainly dependent upon the grade of intestinal metaplasia of the gastric mucosa. Higher frequency of differentiated adenocarcinoma in intestinal cancer than in gastric cancer is additional data supporting this view.

A study of precancerous lesions is of utmost importance, not only from pathological but from clinical standpoints. As was indicated in the results, ulcer cancers were most frequently observed in the mucosal cancer. It is evident, therefore, that the presence of an ulcer, even though it is scared or deeply callotic, plays a promoting role in the occurrence of gastric cancer. The old view[23)] that gastric cancer tend to develop from regenerating epithelia at the margin of the ulcer can be said to be tenable. For the better understanding of exact frequency of the ulcer cancer[25)], however, further detailed and statistical investigations on the mucosal cancer, especially on the cancers developed from acute or scared ulcers, will be requiered.

Konjetzny[15)] pointed out that the intestinal metaplasia of the gastric mucosa is a major histological sign of "chronic gastritis." The present authers have an opinion that the presence of intestinal metaplasia is indispensable for the diagnosis of "chronic gastritis" but chronic inflammatory nature of this disease has not yet been confirmed. Recently, precancerous nature of "chronic gastritis" has drawn much attention in the fields of internal medicine, surgery and pathology[4),5),13),14),22),41),49)]. From the present study, however, no conclusion was obtained that the metaplastic epithelia itself presents a type of precancerous changes. But occasionally, more or less atypical metaplastic epithelia are observed among the ordinary metaplasia, especially in an area of "chronic atrophic gastritis." Difficulty concerning this problem might also be due to rare chances of obtaining such a surgical material, owing to the lack of subjective and objective symptoms. Further clinical, histological and cytological studies will be needed.

## Conclusion

Forty-two cases of the mucosal cancers, in which cancerous lesions were limited within the gastric mucosa, were examined histologically. These cases were obtained from total of 2865 cases of the gastrectomy conducted during past ten years at Yokoyama's Hospital in Nagoya City. The mucosal cancers consisted of 28 cases of ulcer cancer, 5 cases of polyp cancer and 9 cases of superficial spreading erosive cancer without ulcer formation.

Histological pictures of the cancerous tissues were classified into following three types, according to grade of differentiation of the cancerous tissues, namely 1) well-differentiated adenocarcinoma (A type), which was subdivided into papillary, tubular and acinar adenocarcinomas, 2) poorly-differentiated adenocarcinama (B type), having no apparent glandular structures but cord-like structures composed of solid cancerous epithelia and 3) disseminated carcinoma (C type), showing no glandular structures and consisting of dissociated signet-ring cancer cells. Transitional forms from A type to B or B type to C are not infrequently observed.

Histological pictures of the cancerous tissues were compared with those of the cancer-bearing non-cancerous pyloric mucosa and following results were obtained. A type was most frequently seen in cases having moderate or heavy intestinal metaplasia in the pylosic mucosa. All of C type were, on the contrary, seen in cases having no or slight intestinal metaplasia. B type was situated between the above two. Grade of intestinal metaplasia of A–B type was higher than that of B–C type. These results indicate that the histological pictures of the mucosal cancer are dependent on the changes of the mother tissues, especially grade of intestinal metaplasia of the gastric pyloric mucosa.

From histogenetical viewpoints, all of the polyp cancers were classified into A type and almost all of superficial spreading erosive cancers belonged to A type or A–B type. These histological tendencies are however, not observed in the ulcer-cancer. But cancers, showing a pictures of B type, B–C type or C type, were almost all included in the ulcer-cancer.

## Acknowledgement

We wish to express our thanks to Dr. H. Yokoyama, chief of Yokoyama's Hospital, for his encouragement in this study and generous supply ot materials.

This work was supported in part by research grants from the Japanese Ministry of Education.

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## Explanation of Plates V–VII

Figs. 1–4. Macroscopical appearances of the mucosal cancers. The findings are illustrated in Text-Fig. 1. 1; I-19, 2; I-22, 3; I-25, 4; III-3.

Fig. 5. Atrophic lesion composed of atypical metaplastic epithelia. III-3.

Fig. 6. Atrophic lesion composed of atypical metaplastic epithelia. III-9.

Fig. 7. A type cancer (papillary adenocarcinoma). II-1.

Fig. 8. A type cancer (tubular adenocarcinoma) at the margin of the ulcer. I-24.

Fig. 9. A type cancer (tubular adenocarcinoma) within the heavy intestinal metaplasia. III-9.

Fig. 10. A type cancer (tubulo-papillary adenocarcinoma) within the heavy intestinal metaplasia. III-3.

Fig. 11. A type cancer (poorly differentiated adenocarcinoma) in upper half of the figure. I-16.

Fig. 12. A and B types cancer. (poorly differentiated adenocarcinoma and "reticular carcinoma"). Pyloric glands remained under the cancer. I-17.

Fig. 13. B type cancer. Cord-like structures of the cancerous epithelia. The cancerous tissues are desquamated together with mucous exsudates. I-26.

Fig. 14. B type cancer. Cord-like structure of the cancerous epithelia in the mucosa around the ulcer. I-9.

Fig. 15. B type cancer. Cancer cells are gradually separated from cancerous cords. I-17.

Fig. 16. Coexistence of B and C types. I-2.

Fig. 17. C type cancer. Mucous formation of cancer cells is not prominent. I-27.

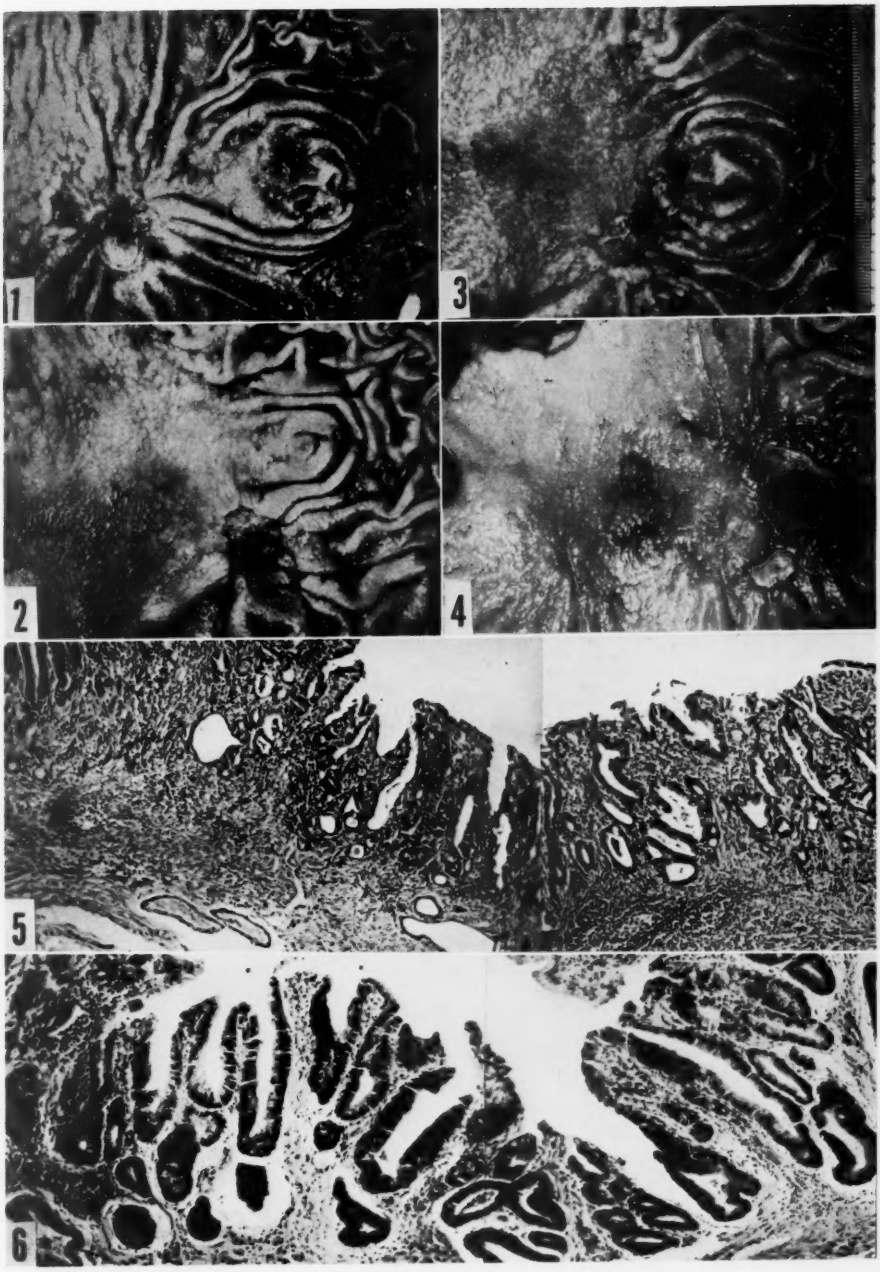
Fig. 18. C type cancer. Upper half of the mucosa is occupied by signet-ring cells. Pseudopyloric corpic glands remained at the bottom of the mucosa. 1-2.

Fig. 19. B type cancer. Cancer cells are arranged in a row and present little glandular structure. I-4.

Fig. 20. B type cancer. Cancer cells are arranged in a row and present no glandular structurs. I-18.

Fig. 21. C type cancer. Gradual transition from B type to C is seen in right half of the figure. I-21.

Fig. 22. C type cancer. Gradual transition from B type to C is seen in left half of the figure. I-18.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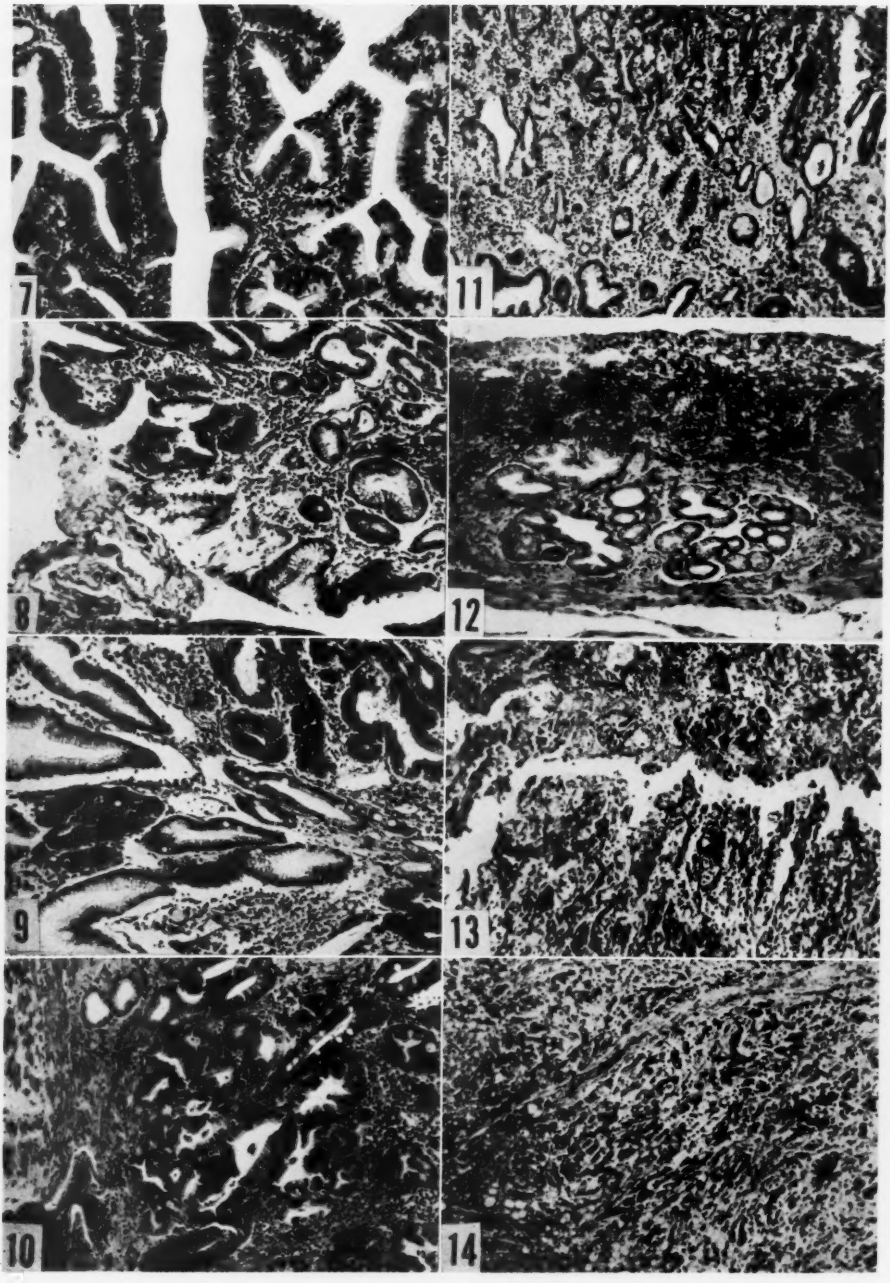
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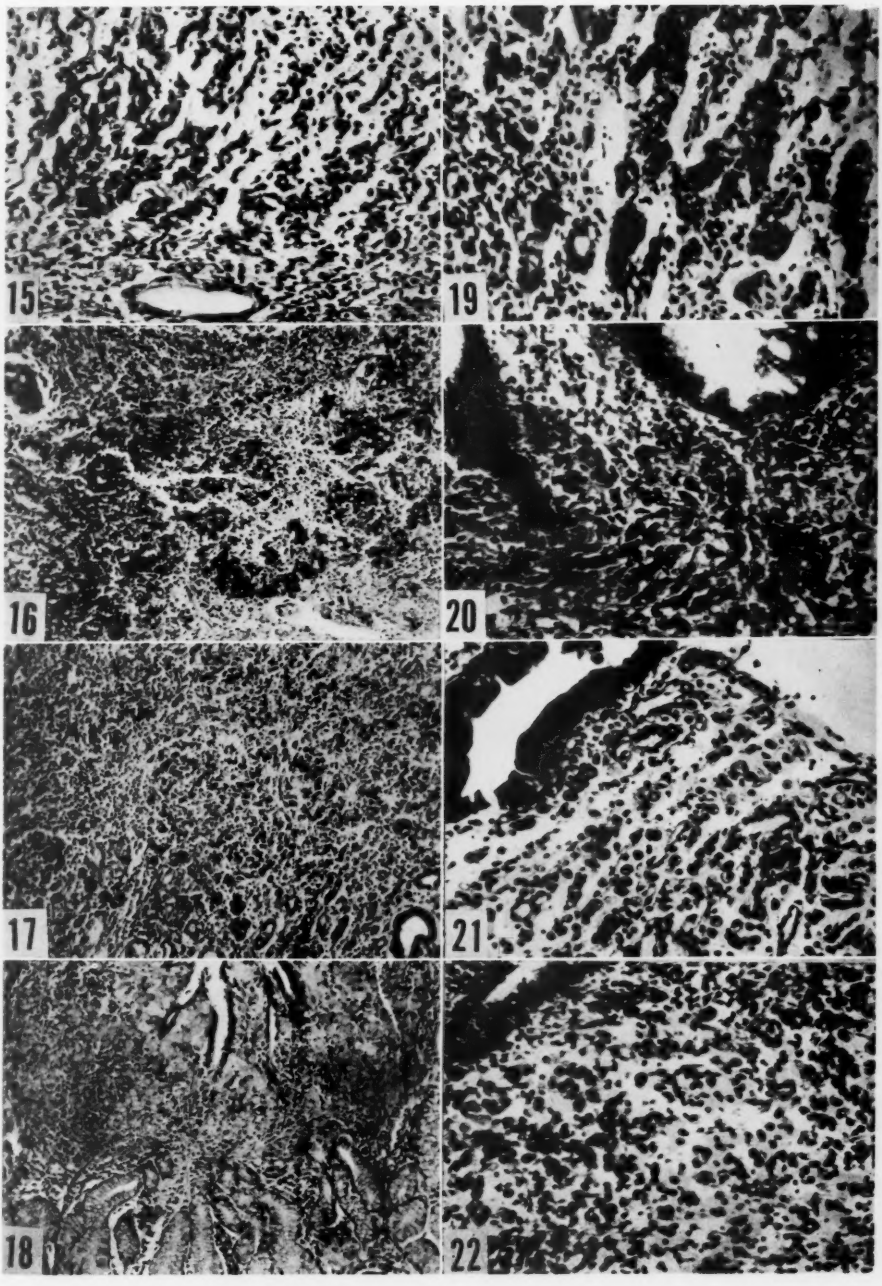
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[GANN, 52, 121~125; June, 1961]

# THE INFLUENCE OF HEMOLYTIC STREPTOCOCCI ON THE BASOPHILIA OF ASCITES TUMOR CELLS

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It was demonstrated by Koshimura et al. (1955) that ascites tumor cells almost completely lost the invasion power to mice, when they are incubated *in vitro* with living hemolytic streptococci. In this case a considerable quantity of streptolysin S was produced (Koshimura *et al.*, 1958). Since streptolysin S production by the streptococci is strongly enhanced by ribonucleic acid (Okamoto, 1940; Bernheimer and Robart, 1948; Bernheimer, 1949), it may be regarded as being produced at the expense of ribonucleic acid contained in the tumor cells.

Hence it was thought worthwhile to investigate cytochemically the changes of ascites tumor cells incubated with living hemolytic streptococci, in order to know whether the loss of invading power of ascites tumor cells to mice is due to the uptake of ribonucleic acid by living streptococci or the action of other factors of the cocci.

The evidence reported herein shows that living hemolytic streptococci principally act in decreasing ribonucleic acid in the cytoplasm of ascites tumor cells.

## Materials and Methods

**Preparation of tumor cell suspension.** Ascites tumor cells collected from mice (weighing 15–20g) inoculated 5 days earlier with approximately $10^7$ sarcoma 180 cells. were washed twice with phosphate-buffered Ringer's solution (a mixture of one part of M/15 phosphate buffer of pH 7.2 and four parts of Ringer's solution). The harvested tumor cells were resuspended at the desired concentration (usually 80–100 million per ml).

**Preparation of bacterial suspensions.** Streptococcus hemolyticus "S", Staphylococcus aureus "209-P", Pneumococcus Type 1 and Escherichia coli "Kosaka" were employed. The bacterial suspension, except that of pneumococcus, was prepared as follows: one drop of a 20 hour-culture of the bacteria to be tested was inoculated into 100 ml of ordinary broth. After incubation at 37° C for 20 hours, the harvested bacteria was washed twice with phosphate-buffered Ringer's solution. Streptococcus and pneumococcus were resuspended in 5 ml, staphylococcus and E. coli in 10 ml of the same solution. In case of pneumococcus, bacterial suspension was prepared from cultures incubated at 37°C for 20 hours in broth containing rabbit serum.

**Procedure.** A half mililiter of tumor cell suspension, 1.0 ml bacterial suspension, 0.5 ml of M/15 phosphate-buffered Ringer's solution were mixed and incubated at 37° C for periods of 10, 20, 30, 60, 90, 120 minutes, etc. In every experiment the mixture of tumor cell suspension and bufferd Ringer's solution were similarly treated as control at the same time.

**Ribonuclease.** Crystalline ribonuclease which was obtained from Wako Pure Chemicals Ind., Ltd., as a commercial preparation was added to the mixture of tumor cell suspension and buffered Ringer's solution at 0.025 and 0.25 per cent.

**Streptolysin S.** Purified sample of streptolysin S (minimum hemolytic concentration=1; 20 million) was kindly supplied from Prof. S. Koshimura, Research Institute of Tuberculosis, Kanazawa University, Kanazawa, Japan. It was added at 0.005 and 0.05 per cent.

**Histological preparations.** All rapidly air-dried smears from the mixtures were fixed for 3 minutes in 10 per cent neutral formalin without delay, washed in distilled water for 5 minutes and stained with methyl green-pyronine and by Feulgen nuclear reaction staining techniques.

## Results

Figs. 1–5 show the results of incubating tumor cells. The streptococci were soon deposited on the cell surface. However, it was morphologically not decided whether the streptococci penetrate the tumor cell wall or whether it is only deposited on the cell surface. Smears from tumor cells incubated for 30 minutes indicated a decrease of basophilia in cytoplasm of the cells on which surface the streptococci were deposited (Fig. 1). After incubation for 60 minutes, not only marked decrease of basophilia in cytoplasm of all cells occured, but cytoplasm nearly vanished and swollen free nuclei remained (Fig. 2). A decrease in staining intensity of desoxyribonucleic acid in nucleus was generally not observed at this time. After 90–120 minutes, nuclei with diminished staining intensity of desoxyribonucleic acid gradually increased (Fig. 3). The staining intensity of ribonucleic acid in nucleoli was maintained.

Control cells incubated in buffered Ringer's solution always showed more intense basophilia in cytoplasm and desoxyribonucleic acid staining in nucleus, even after incubation for 120 minutes (Figs. 4, 5).

The changes above described were never demonstrated when the tumor cells were similarly incubated with suspension of the hemolytic streptococcus preheated at 56° C for 60 minutes, pneumococcus, staphylococcus aureus or Escherichia coli.

The treatment of tumor cells with crystalline ribonuclease caused no decrease in the cytoplasmic basophilia of ascites tumor cells.

Streptolysin S (0.05 per cent) produced swelling of cells and decrease of cytoplasmic

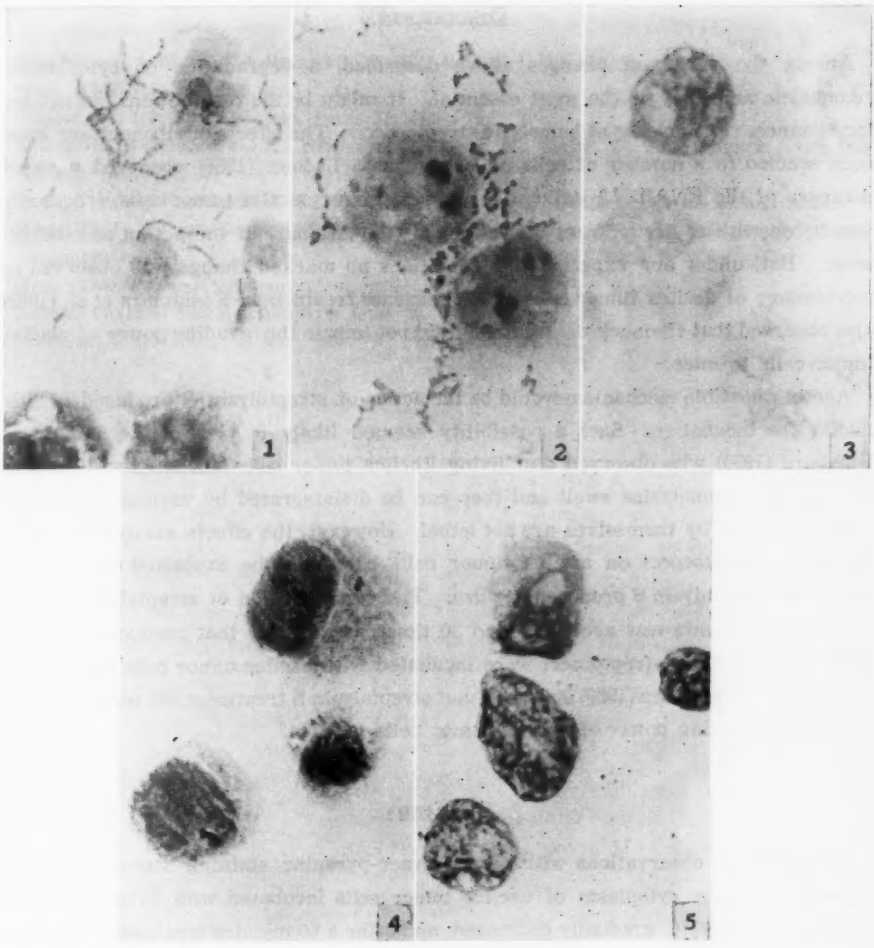


Fig. 1. Sarcoma 180 cells incubated with hemolytic streptococci. Incubation time 30 minutes. Methyl green-pyronine staining.
Fig. 2. Same. Incubation time 60 minutes. Methyl green-pyronine staining.
Fig. 3. Same. Incubation time 120 minutes. Feulgen staining.
Fig. 4. Control. Incubation time 120 minutes. Methyl green-pyronine staining.
Fig. 5. Control. Incubation time 120 minutes. Feulgen staining.

basophilia, but a few cells with basophilia in cytoplasm remained even after incubation for 120 minutes.

## Discussion

Among the series of changes above described, a degradation of cytoplasmic ribonucleic acid may be the most essential. It might be the result of enzyme action, for instance, ribonuclease of hemolytic streptococci. The effects of ribonuclease have been studied in a number of cells or organisms. Ledoux (1956) observed a rapid decrease of the RNA/DNA ratio with young and very active tumor cells, frequently associated with a disruption of the cell during the last part of the action of ribonuclease. But, under our experimental conditions no marked change was observed in morphology of ascites tumor cells by ribonuclease treatment. Koshimura *et al.* (1960) also observed that ribonuclease treatment did not impair the invading power of ascites tumor cells to mice.

Another possible mechanism would be the action of streptolysin S produced *in vitro* during the incubation. Such a possibility seemed likely in view of the findings of Ginsburg (1959) who observed that living Ehrlich tumor cells incubated with various streptococcal hemolysins swell and they can be disintegrated by various proteolytic enzymes which by themselves are not lethal. However, the effects exerted by living hemolytic streptococci on ascites tumor cells could not be explained only by the action of streptolysin S produced *in vitro*. The concentration of streptolysin S used in our experiments was about 300 and 30 times as much as that produced *in vitro*, when the hemolytic streptococci were incubated with ascites tumor cells (Koshimura *et al.* 1958). Koshimura (1960) observed that streptolysin S treatment did not materially affect the invading power of ascites tumor cells to mice.

## Summary

Cytochemical observations with methyl green-pyronine staining showed that the basophilia in the cytoplasm of ascites tumor cells incubated with living hemolytic streptococci at 37°C gradually decreased, and after a 60 minutes treatment cytoplasm vanished and swollen free nuclei remained. After incubation for 90–120 minutes, the staining intensity of desoxyribonucleic acid by Feulgen technique in nuclei more or less decreased. Heat-killed streptococcus, staphylococcus, pneumococcus and Escherichia coli were ineffective in decreasing the cytoplasmic basophilia of the tumor cells. Ribonuclease treatment of tumor cells produced no decrease in cytoplasmic basophilia. The effects of living hemolytic streptococci on ascites tumor cells, however, is not explained by the action of streptolysin S produced *in vitro*.

The authors with to express their thanks to Prof. T. Ishikawa and Prof. H. Okamoto for valuable suggestion and encouragement, and to Prof. S. Koshimura for kind help in experiments.

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# ON THE RELATION BETWEEN ELECTRONIC STRUCTURE AND CARCINOGENIC ACTIVITY OF URETHAN (ETHYLCARBAMATE) AND RELATED COMPOUNDS

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## INTRODUCTION

The study of the nature of the interaction between tissue constituents and carcinogens is fundamental to an understanding of the genesis of chemically induced tumor, and the knowledge on the submolecular level has begun to open the way to unfold the mystery surrounding the cancer induction. In this respect the study of the relationship between the electronic structure and the carcinogenic activity may be one of the heuristic methods for the elucidation of the mechanism of carcinogenesis.

In the previous papers (1-4, 25) the present authors found an intimate relation between the electronic distribution and the carcinogenic activity of important series of carcinogenic compounds such as polycondensed aromatic hydrocarbons, azo compounds and 4-nitroquinoline-N-oxide derivatives, and gained a clue to obtain knowledges about the positions in the carcinogens to be attacked by tissue constituent and the nature of the reaction of the carcinogenic action of these compounds.

In the present paper, the electron distributions of urethan and its related compounds are obtained using the frontier electron method which has been established by the present authors (5-8), and the results are compared with the experimental data of carcinogenic activity.

A distinct relation is found between the frontier electron distribution for nucleophilic attack at the position of carbonyl carbon and the carcinogenic activity of these compounds. This finding may add a support to the postulation which has been insisted on by the present authors that the attack of carcinogen on the nucleophilic or electron rich center in the body might be involved in the genesis of tumors. In relation to this view, the carcinogenic activities of miscellaneous chemical carcinogens such as various kinds of alkylating agents, acetylaminofluorene and its derivatives, dulcin, isonicotinylhydrazine, etc., whose carcinogenicity are hardly explained by the K-region theory (24), are discussed, and a unified explanation on the carcinogenesis of these carcinogens is given from the standpoint of nucleophilic reactivity of these compounds.

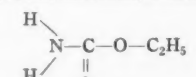
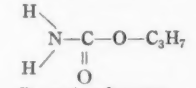
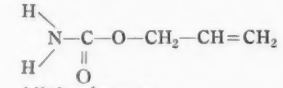
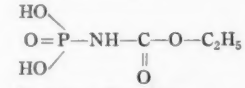
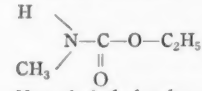
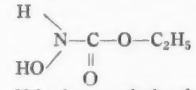
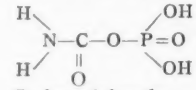
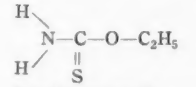
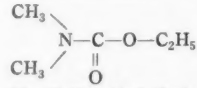
## The Carcinogenicity of Urethan Compounds

Before proceeding with the investigation, the authors have paid an attention to the fact that the carcinogenic activity of urethan and its related compounds is somewhat different from those of the aromatic hydrocarbons, azo dyes, 4-nitroquinoline-N-oxides, etc., which are the typical examples of chemical carcinogens. Since the first report of Nettleship *et al.* (9) regarding the incidence of lung tumor in mice, numerous experiments indicating the carcinogenicity of urethans have been carried out not only on the mice but also on the rat. And the tumors developed include the adenoma of lung (9–14, 21), papilloma of skin (15–18) or fore stomach (19) and hepatoma (20). Although there have been accumulated data on the carcinogenicity of urethan, almost all of these tumors have been identified as not malignant. In this respect, the effect of urethan and related compounds on the test animal seems not entirely the same as that of the aromatic hydrocarbons, etc., which have been usually called the chemical carcinogens. Many researchers, however, have treated the urethans as a group of chemical carcinogens in spite of the benign nature of tumors developed by these compounds (9–22), and they appear to have recognized implicitly that tumor induction by urethan is analogous to those of the typical carcinogens, though the detailed discussion has not been given on this point. Recently Nakahara and Fukuoka (23) discussed the mechanism of tumor induction and presented a scheme by which the induction of malignant and benign tumors could be explained. According to this theory the difference in the development of the malignant and benign tumors is not qualitative but is quantitative. In view of these considerations, the treatment of urethan compounds from the standpoint of chemical reactivity may add useful knowledge to the mechanism of carcinogenesis.

## Results and Discussions

The electronic distribution at each position in the molecule of urethan and its related compounds is compared with the experimental carcinogenicity of these compounds and it was found that the approximate superdelocalizability for nucleophilic attack, $S'^{(N)}$, at the carbonyl carbon have an intimate correlation with the carcinogenic activity. The experimental data are the results of Berenblum *et al.* (21) on the male and female mice of the Swiss strain and the degrees of activity of these compounds are shown by plus and minus signs, in the case of initiating action on skin and carcinogenic action on the lung, respectively. It is needless to say that the more the plus signs has the compound, the more activity is the compound in carcinogenicity, and the compound having feeble activity is shown by the $\pm$ sign. The signs in the third and fourth columns in Table 1, is taken from the paper published by Berenblum *et al.* (21). It is of interest that the degrees of activities of these

Table 1. Relation between the electron distribution and carcinogenic activity of urethan and its related compounds.

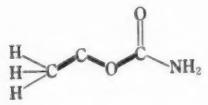
| Compound | *Approximate superdelocalizability, $S'_{(N)}$ at carbonyl carbon | Initiating action on skin | Carcinogenic action on the lung |
|---|---|---|---|
|  Ethylcarbamate (Urethan) | 1.0322 | ++++ | ++++ |
|  Propylcarbamate | 1 0322 | + | + |
|  Allylcarbamate | 1.0318 | + | − |
|  Urethan phosphate | 1.0254 | ± | ± |
|  N-methylethylcarbamate | 0.9798 | ++ | ++ |
|  N-hydroxy-ethylcarbamate | 0.9792 | +++ | ++ |
|  Carbamylphosphate | 0.9733 | ± | − |
|  Xanthogenamide | 0.9242 | ± | − |
|  N: N-dimethylethylcarbamate | 0.9203 | − | − |

* The coulomb integral for nitrogen, oxygen and phosphorus atoms, and methyl group are the same for ones used in the previous papers (33, 34). For hydroxyl group and sulfur atom, $\alpha+\beta$ and $\alpha+0.8\beta$ respectively, are used.

compounds on skin and on the lung are entirely the same, and this might suggest that the nature of the interaction of urethan compounds with body constituents was similar to each other in both cases.

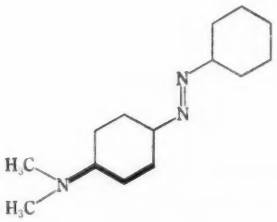
As is seen in Table 1, the compound whose $S'^{(N)}$ value at the carbonyl carbon is larger than the threshold value, 0.979, has carcinogenic action on the lung as well as on skin. On the other hand, the compound whose $S'^{(N)}$ value is under the threshold is non-carcinogenic. In spite of the equality of the theoretical index of ethyl and propyl carbamic esters, carcinogenic activity of ethyl compound is extremely large. This might be the result of the most favorable sterical situation with regard to the fitness of the compound to the receptor in the body.

At the present stage of knowledge it is difficult to determine the sterically most suitable condition for carcinogenicity. However, the fact that the ethyl carbamate has an analogous structure to aromatic hydrocarbons, azo dyes, etc. is of interest. The situation is illustrated in Fig. 1.

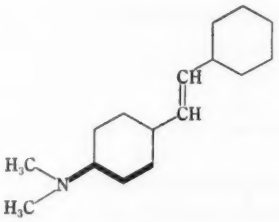


URETHAN

3, 4-BENZPYRENE



DAB



4-DIMETHYLAMINOSTILBENE

Fig. 1. Sterically similar structures (the part of heavy line) of urethan and other carcinogens.

The importance of the carboethoxy portion for carcinogenicity of urethan compounds was pointed out by Berenblum *et al.* (21).

carbamyl portion

$$NH_2—CO—O—C_2H_5$$

carboethoxy portion

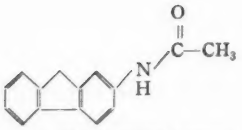
If the carbonyl carbon and the methyl carbon which is, in general, reactive toward various kinds of reagent are considered, the relation between these two active centers bears a resemblance to that of the principal and subsidiary carcinogenophores of aromatic hydrocarbons and also to that of the two reactive centers of azo compounds and stilbene derivatives. In this connection the following fact is worthy of attention : that is, the lung tumor induced by treatment of urethan was histologically of the same type as that developed after treatment with carcinogenic hydrocarbons, and the hepatomas also resembled those produced by DAB. (20).

## Nucleophilic Reactivity and Carcinogenic Activity

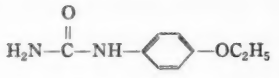
Polycondensed aromatic hydrocarbons and azo compounds have been two main series of chemical carcinogens and extensively studied from different points of view. But at the present time a large number of chemical compounds belonging to many different classes have been shown to be tumor-producing and the researchers are fumbling for the diversity of too many different types of chemical carcinogens. Thus, the carcinogenicity of 4-nitroquinoline-N-oxide, 2-acetylaminofluorene and its derivatives, various kinds of alkylating agents such as nitrogen mustards, etyleneimine derivatives, trimethylolmelamine, epoxide, methosulfonate, etc., and urethan compounds, dulcin, thiourea, isonicotinylhydrazine, semicarbazid, pyrazinamide, etc., has been found, and the unified explanation of the carcinogenicity of various kinds of such chemicals has been sought for.

The K-region theory has been proposed in order to correlate the carcinogenic activity with the chemical constitution but the validity of the theory is limited to such a compound as one in which the K-region exists (24).

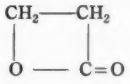
The present authors have paid an attention to a common nature in chemical carcinogens by which the unified explanation of the carcinogenicity of these compounds may be possible. Namely, from the consideration of the electronic distribution the authors concluded that the carcinogenicity of the aromatic hydrocarbons, azo compounds, 4-nitroquinoline-N-oxide and its related compounds, and 4-dimethylaminostilbene derivatives is intimately connected with the reactivity at some positions in the molecule towards nucleophilic reagents or electron rich centers in the body. As has been pointed out in the previous section, the carcinogenicity of urethan compounds also is associated with the nucleophilic reactivity of the carbonyl carbon which is deficient in the electron density. It is of interest that acetylaminofluorenes, dulcin, propiolactone, isonicotinylhydrazine, formamide, pyrazinamide and thiourea have an active carbonyl carbon or sulfur which is reactive toward nucleophilic reagents.



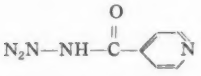
2-Acethylaminofluorene



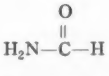
Dulcin



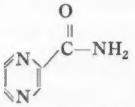
Propiolactone



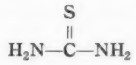
Isonictinylhydrazine



Formamide



Pyrazinamide



Thiourea

In connection with this view, the following fact pointed out by Mori *et al.* (32) is of interest. That is, the compounds which are capable of inducing a pulmonary tumor on mice, have a remarkable similarity in molecular structure to one another. Thus, isonicotinylhydrazine, urethan, semicarbazid, pyrazinamide, etc., which induce pulmonary tumors all contain carbamyl group ($NH_2-\underset{\underset{O}{\|}}{C}-$ ).

Furthermore, the biological activity of alkylating agents has been assumed to originate from the alkylation of the nucleophilic center in the body. Thus, the carcinogenicity of various kinds of carcinogens could be explained from the point of view of the nucleophilic reactivity.

Concerning the anti-tumor activity of aromatic compounds such as 4-dimethylaminostilbene, the present authors presented the postulation that the carcinostatic activity of these compounds had an intimate connection with nucleophilic reactivity at position 2, the dimethylaminonitrogen and the $\alpha$-carbon, and a strong resemblance between the carcinogenic and carcinostatic activities was pointed out.

Granting that the interaction of the chemical compound with a nucleophilic center in the body is concerned with the incidence and inhibition of tumors, some interrelations may be expected between the carcinogenicity or carcinostatic activity and cholinesterase (ChE) inhibition, because ChE has been supposed experimentally to have a nucleophilic center (so called the esteratic site) and an anionic site (30, 31).


Fig. 2. Active centers in cholinesterase (according to wilson et al. (30, 31)).

(see Fig. 2). In this connection, the fact that the anticancer agents such as TEM, TEPA, nitrogen mustards, etc., have shown an inhibitory effect on the enzymatic action of ChE. (26-28) is of interest. On the other hand, paraoxon which is a well-known ChE inhibitor and consequently has been used widely as an insecticide was

found to inhibit the growth of tumor (29). In view of the similarity in reaction nature of carcinogenic and carcinostatic actions (25), the carcinogenic activity of paraoxon may be anticipated.

## Active Centers in the Body

The postulation that the nature of reaction of carcinogenesis may be nucleophilic leads us to the knowledge about the group or groups serving as the active center in the body. As the nucleophilic center various kinds of groups are considered. These groups are e.g. sulhydryl, hydroxyl, carboxyl, amino, imidazole ring, etc. in protein. In the nucleic acid molecule, amino groups, hydroxyl groups, active nitrogen atoms, etc., exist, and DPN, TPN or FAD also has nucleophilic groups in the molecules. To determine which group is truely concerned with the genesis of tumors is difficult in view of the poor knowledge that we have at present about the relation between the structure and the function of protein and nucleic acid. But it may be worth noticing that the compounds such as nitrogen mustards, formaldehyde, etc., which are very reactive toward nucleophilic reagents and consequently seem to react easily with the above-stated groups in the body have shown not to be so strong a carcinogenicity. On the other hand, aromatic hydrocarbons and azo dyes whose nucleophilic activity are below that of nitrogen mustards or formaldehyde have larger carcinogenicity than the latter compounds. This fact might suggest that all of the active groups in the body are not involved in the carcinogenesis. Certain specific groups or atoms like the esteratic site of ChE, satisfying some specific steric conditions, might take part in the induction of tumors.

## Summary

The electron distribution of urethan and its related compounds are obtained using the frontier electron method which has been established by the present authors as one of the molecular orbital methods to discuss chemical reactivity and the results are compared with those of carcinogenesis experiments.

The frontier electron distribution at the carbonyl carbon shows a parallelism with the degree of carcinogenicity of these compounds. This relation leads to the conclusion that the interaction between the carbonyl carbon and the nucleophilic active center in the body might play an important role in the induction of tumor by these compounds and this finding may be an additional support to our postulation that the attack of carcinogen on the nucleophilic center in the body might be involved in the early stage of carcinogenesis.

The sterical resemblance between the urethan and other carcinogens is pointed out and some discussions are made on the active nucleophilic center in the body in connection with the cholinesterase inhibition.

Financial support for this work has been received in part from the Education Ministry of the Japanese Government.

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# STUDIES ON THE PYRUVATE METABOLISM DURING HEPATOCARCINOGENESIS: IN VIVO METABOLISM OF PYRUVATE-2-$C^{14}$ IN THE LIVER OF 3'-ME-DAB FED RATS

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Many works have been done with regard to the metabolic changes during carcinogenesis. In the carcinogenesis by azo dyes, various enzymes in various stages were assayed with results demonstrating steady decrease in some and no remarkable changes in others.[1,2,3,4,5,6,7,8,9] The significance of these changes, as well as the rôle of metabolim in carcinogenesis, however, are not yet fully understood. Gradual decrease in respiration and increase in glycolysis in the course of azo dye administration were reported by Dessi.[10] Although the high glycolysis in tumor cells was confirmed by many workers[11,12] since Warburg,[13] the concurrent inhibition of respiration (or oxidation) is now in dispute. In fact, as Weinhouse indicates,[14] tumor does not exhibit a deficient oxidation but only a high aerobic glycolysis in the in vitro conditions. The observation of Wenner et al.[15], representing no significant difference in the enzymes of TCA cycle between normal and tumor tissues, accounts for this fact. On the contrary, the data of Busch and his collegues[16,17] suggest the lowered oxidation in the tumors in intact animals. They studied *in vivo* metabolism of acetate-1-$C^{14}$ and pyruvate-2-$C^{14}$ using transplantable tumors with the results that tumors in situ are virtually incapable of oxidizing acetate in contrast with the rapid utilization to glutamate in most normal tissues. They also found the strongly diminished incorporation of the isotope of the pyruvate-2-$C^{14}$ into the TCA cycle-related amino acids in the tumor tissues as compared with many control tissues. Pyruvate injected was mostly converted to lactate as a hydrogen acceptor. These results imply that the inhibition of oxidation do exist as well as high anaerobic glycolysis in the tumor in its physiological state. Some studies, *in vitro*, of Strength and Seibert[18] and of Allard et al.[19] on electron transport system in tumors seem to be compatible with this. This discrepancy may be ascribed to some controling mechanisms operating in situ. In the experiments *in vitro*, however, the amount and the state of co-enzymes, the amount of substrate available and the spacial distribution of these components, including the enzyme itself, in cells or in cell particles must be taken into account. From this point of view the experiment *in*

*vivo* is, even now, advantageous in that it can look into the metabolism of cell or tissue in its most natural state.

The author, in consideration of these circumstances, wanted to extend the studies of Busch to see metabolism during carcinogenesis. So, the primary objective of this study was to comfirm the "metabolic pattern" of the tumor in the 3'-Me-DAB induced hepatoma, and to follow up the change in the pattern during hepatocarcinogenesis. For, it is a question whether these changes arise gradually during carcinogenesis through precancerous stage, or abruptly when the cancer cells emerge. The present paper describes the results obtained with 3'-Me-DAB fed rats in the course of hepatocarcinogenesis, comparing the injury with those in carbon tetrachloride poisoning and choline deficient fatty liver.

## Materials and Methods

**Experimental animals. Carcinogenic experiments:** Male albino rats weighing about 200 g were fed rice containing 0.06 per cent 3'-Methyl-4-dimethyl-aminoazobenzene (3'-Me-DAB). Serious malnution and vitamin deficiency were prevented by administering small amount (about 3 gm) of dried fish and some raw vegetebles 2 or 3 times a week. The experiments were performed at the ends of the following three divided periods:

I. Two weeks feeding of 3'-Me-DAB, when the amount of the protein bound dye in the liver is maximum (Early stage).

II. Two or three months feeding of 3'-Me-DAB, when the livers showed various stages of liver cirrhosis or precancerous state (Late stage).

III. One month feeding of standard diet after three months feeding of 3'-Me-DAB. In this experiment, the animals were used only when liver tumors were palpated.

**Carbon tetrachloride poisoning:** In this experiment, 0.2 ml of carbon tetrachloride per 100 g body weight was injected intramuscularly in a sound albino rat in the form of 10% olive oil solution. Four hours later, the animals were injected with the tracer.

**Choline deficient fatty liver:** In this experiment, male infant albino rats were maintained on a choline deficient diet containing 54 per cent Starch, 10 per cent casein, 15 per cent sucrose, 10 per cent Crisco, 4 per cent McCollum salt, 2 per cent agar, 5 per cent cod liver oil, and adequate vitamin mixture consisting of each 500$\gamma$ of thiamine, riboflavin and pyridoxine, each 2 mg of nicotinamide and calcium pantothenate. At the period of three days and thirty days, the animals were used for the experiment.

**Treatment of animals and tissues.** The animals were given the diet and water ad libitum till the time of experiment. They were injected 5 microcuries of sodium pyruvate-2-$C^{14}$ (specific adtivity: 17.5 microcuries/mg) intravenously from the tail

vain. 3 and 8 minutes after the injection, the animals were decapitated and exang uinated from the neck. Then the livers were excised as soon as possible and immediately frozen in acetone dry ice. The blood was deproteinized in 0.33 N perchloric acid and used for the determination of the count in blood. After weighing, the tissuc (liver or liver tumor) was homogenized in 0.6 N perchloric acid and centrifuged. The supernatant solution was nautralized by 2 N potassium hydroxide and stored in a refrigerator overnight. Precipitated potassium perchlorate was removed by filtrabion, and a part of the supernatant was used for the determination of the total count of the tissue. An aliquot of the extract, derived from 1 g of tissue was alkalified to pH 12 by potassium hydroxide, and washed into the column described below. Glycogen content was measured by the anthrone method as well as the incorporation of the isotope into after repeated purification.

**Anion exchange chromatography.** According to Busch, the main metabolic fate of injected pyruvate is the reduction to lactic acid, the amination to alanine and the distribution to the members of TCA cycle and related amino acids. The author, after confirming this fact, used almost the same method as Busch et al. had described in the previous reports,[17,20,21] with some modifications. So, the chromatographic procedure will be described only briefly.

Preparation of the column: Dowex-1, x-4 (200-400 mesh) was conditioned as usual and turned to acetate form by sodium acetate. 10 ml of the resin was packed into a glass tube, 1 cm in diameter and 25 cm in length. Four columns were joined to a distributor which was connected to a mixing lask contaning 800 ml of distilled water. The mixing flask receives acid solvent through rubber tube from a reservoir placed about 1 meter high. Four samples were chromatographed at the same time using this apparatus.

Elution: After spotting the samples to the columns, gradient elution was performed as follows:

i) Number of the column: 4 (1×25 cm)
ii) Contents of the mixer: 800 ml of distilled water
iii) Speed of flow: 0.8-1.0 ml/min/column
iv) Collection of effluent: 2 ml/fraction
v) Total number of fraction: 80/column
vi) Change of solvent in reservoir:

| Fraction number | Solvent in reservoir |
|---|---|
| 1-32 | 4 N acetic acid |
| 33-42 | 6 N acetic acid |
| 43-80 | 6 N formic acid |

By this method, the mixture of alanine, glutamic acid, aspartic acid, lactic acid, succinic acid and malic acid was separated in discrete peaks as Figure 1. The

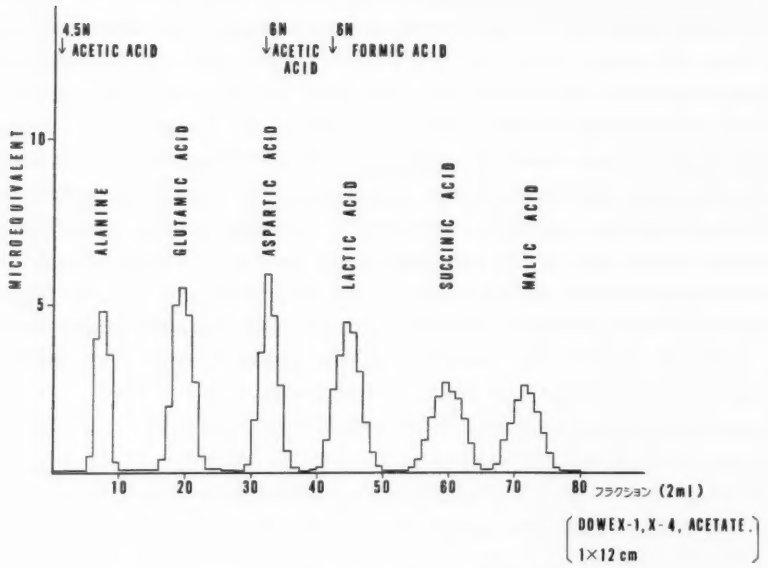


Fig. 1. Anion Exchange Chromatogram of Standard Samples.

reproducibility was also excellent.

Quantitization: After the elution, all the fraction except those of lactic acid, which are checked by the Feigl's "Spot test",[22] were dried in a desiccator for 30–40 hours under the vacum condition of 20 mm Hg in 37°C. Scaled sodium hydroxide and dried calcium chloride were placed in the desiccator. Alanine was determined by the ninhydrine colorimetry of Moore and Stein. Lactic acid was by the method of Barker and Summerson. The other compounds were titrated by 0.01 N sodium hydroxide after desiccation. The titration value of glutamic and aspartic acid corresponded closely to the ninhydrine colorimetry. The recoveries of these substances reached 95–100 per cent.

Identification: Each substance was identified by paper chromatography. Phenol: water (100 : 20) with 3 per cent ammonium hydroxide solution in the chamber and the supernatant of butanol: acetic acid: water (4 : 1 : 5) were used respectively in amino acids and organic acids.

Examination with liver extract: Alanine, glutamic acid, aspartic acid and lactic acid were determined correctly in the liver extract. Succinic and malic acid, however, were not titrated correctly owing to the contamination of nucleotides, sugar phosphates, amino acids and other acidic compounds. Accordingly the specific activities of these two substances were not calculated.

Measurement of Radioactivity: An aliquot of each sample was pipetted onto a

Table 1. Metabolism of sodium pyruvate-2-$C^{14}$ in livers of rats in several conditions and in hepatoma.

| | | Control | | | 3′-Me-DAB FED (Early) | | | 3′-Me-DAB (Late) | | | 3′-Me-DAB Induced Tumor | | | Adjacent Liver | | | $CC_4$ Treated (Acute) | | | Choline-DEF. (Early) | | | Choline-DEF. (Late) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | c.p.m. | Am | Sp | c.p.m. | Am | Sp | c.p.m. | Am | Sp | c.p.m. | Am | Sp | c.p.w. | Am | Sp | c.p.m. | Am | Sp | c.p.m. | Am | Sp | c.p.m. | Am | Sp |
| 3 Minutes-Experiment | Alanine | 2250 | 14.4 | 156 | 2410 | 14.9 | 162 | 885 | 15.2 | 58.2 | 61 | 3.94 | 15.5 | 683 | 12.4 | 55.1 | 28 | 9.35 | 2.99 | 522 | 15.1 | 101 | 1440 | 14.4 | 100 |
| | Glutamic | 927 | 5.05 | 184 | 685 | 3.85 | 178 | 200 | 2.40 | 83.2 | 24 | 3.40 | 7.06 | 169 | 3.40 | 49.7 | 25 | 1.75 | 14.3 | 822 | 5.00 | 164 | 463 | 4.25 | 109 |
| | Aspartic | 1690 | 5.80 | 291 | 1470 | 4.25 | 346 | 155 | 1.55 | 100 | 5 | 1.15 | 4.35 | 88 | 1.75 | 50.2 | 25 | 2.50 | 10.0 | 387 | 3.40 | 114 | 807 | 5.05 | 160 |
| | Lactic | 2322 | 2.25 | 1030 | 2520 | 1.66 | 1520 | 1890 | 6.50 | 291 | 396 | 12.1 | 32.8 | 2830 | 5.64 | 503 | 3000 | 4.48 | 670 | 3180 | 2.58 | 1230 | 1357 | 3.02 | 450 |
| | Succinic | 336 | | | 330 | | | 180 | | | 10 | | | 68 | | | 24 | 5.00 | 4.80 | 292 | | | 300 | | |
| | Malic | 656 | | | 689 | | | 242 | | | 58 | | | 215 | | | 170 | 2.75 | 61.8 | 527 | | | 496 | | |
| | N-EFF. | 1152 | | | 736 | | | 450 | | | 126 | | | 130 | | | 231 | | | 732 | | | 440 | | |
| | Recovered in Chromat. | 9333 | | | 8847 | | | 4002 | | | 680 | | | 4183 | | | 3507 | | | 7462 | | | 5303 | | |
| | Undet. | 5267 | | | 2953 | | | 798 | | | 720 | | | 1117 | | | 2147 | | | 1138 | | | 1147 | | |
| | Total | 14600 | | | 11800 | | | 4800 | | | 1400 | | | 5300 | | | 5650 | | | 8600 | | | 6450 | | |
| | Blood | 6000 | | | 6400 | | | 5280 | | | 4320 | | | | | | 13080 | | | 2800 | | | 6680 | | |
| 8 Minutes-Experiment | Alanine | 1292 | 15.0 | 86.0 | 1080 | 16.9 | 64.0 | 691 | 19.8 | 34.8 | 193 | 7.02 | 27.5 | 476 | 5.46 | 137 | 40 | 15.4 | 2.60 | 256 | 13.8 | 18.5 | | | |
| | Glutamic | 1168 | 3.70 | 31.6 | 374 | 5.10 | 74.8 | 192 | 3.30 | 58.2 | 191 | 2.65 | 72.1 | 629 | 3.50 | 180 | 28 | 1.60 | 17.5 | 220 | 3.90 | 56.5 | | | |
| | Aspartic | 701 | 7.10 | 99.0 | 92 | 1.50 | 61.3 | 131 | 2.10 | 62.4 | 49 | 1.60 | 30.7 | 139 | 1.45 | 95.8 | 34 | 3.05 | 11.2 | 163 | 3.10 | 52.5 | | | |
| | Lactic | 4650 | 4.43 | 1050 | 1510 | 5.16 | 293 | 918 | 5.13 | 179 | 1340 | 10.8 | 124 | 4000 | 2.76 | 1450 | 2080 | 8.15 | 225 | 507 | 2.83 | 179 | | | |
| | Succinic | 675 | | | 143 | | | 0(?) | | | 49 | | | 331 | | | 36 | 3.05 | 11.8 | 187 | | | | | |
| | Malic | 968 | | | 255 | | | 113 | | | 165 | | | 450 | | | 214 | 1.28 | 167 | 148 | | | | | |
| | N-EFF. | 800 | | | 638 | | | 512 | | | 162 | | | 463 | | | 120 | | | 605 | | | | | |
| | Recovered in Chromat. | 10254 | | | 4092 | | | 2557 | | | 2149 | | | 6758 | | | 2552 | | | 2086 | | | | | |
| | Undet. | (−754) | | | 78 | | | 1193 | | | 151 | | | 2142 | | | 1998 | | | (−336) | | | | | |
| | Total | 9500 | | | 4170 | | | 3750 | | | 2300 | | | 8900 | | | 4550 | | | 1750 | | | | | |
| | Blood | 2760 | | | 1870 | | | 4340 | | | 2240 | | | | | | 9480 | | | 1440 | | | | | |

Each value is the average for two experiments of the same type. c.p.m.: count per minute, Am: amount in mioromole/gm wet weight of tissue, Sp: specific activity, N-EFF.: neutral effluent, the portion of the liver extract whch was not attached to the resin by the condition mentioned in the text. It will contain glucose and other neutral or basic substances., Undet.: undetermined, calculated by (Total)-(Recovered in Chromt.). It will contain the count of pyruvate-2-$C^{14}$ itself. A few instances in wich this difference was minus (presented in parentheses) would be due to the technical error., Total: c.p.m. of the perchloric expract from 1 g of tissue., Blood: c.p.m. of perchloric extract from 1 ml of blood.

Table 2. Distribution of the isotope of sodium pyruvate-2-$C^{14}$ to each substance.

| | | Control | 3′-Me-DAB-FED (Early) | 3′-Me-DAB (Late) | 3′-Me-DAB Induced Tumor | Adjacent Liver | $CCl_4$ Treated (Acute) | Choline-DEF. (Early) | Choline-DEF. (Late) |
|---|---|---|---|---|---|---|---|---|---|
| 3 Minute-Experiment | Alanine | 15.4% | 20.4 | 18.4 | 4.4 | 12.9 | 0.5 | 17.7 | 22.3 |
| | Glutamic | 6.4 | 5.8 | 4.2 | 1.7 | 3.2 | 0.4 | 9.6 | 7.6 |
| | Aspartic | 11.6 | 12.4 | 3.2 | 0.4 | 1.7 | 0.4 | 4.5 | 12.5 |
| | Lactic | 15.9 | 21.4 | 39.4 | 28.3 | 53.5 | 53.0 | 37.0 | 21.0 |
| | Succinic | 2.3 | 2.8 | 3.8 | 0.7 | 1.3 | 0.4 | 3.4 | ŧ.7 |
| | Malic | 4.5 | 5.8 | 5.0 | 4.1 | 4.1 | 3.0 | 6.1 | 7.7 |
| | N-EFF. | 7.9 | 6.3 | 9.4 | 9.0 | 2.5 | 4.1 | 8.5 | 6.8 |
| | Recovered in Chromat. | 64.0 | 74.9 | 83.4 | 48.6 | 79.2 | 61.8 | 86.8 | 82.2 |
| | Undet. | 36.0 | 25.1 | 16.6 | 51.4 | 20.8 | 38.2 | 13.2 | 17.8 |
| | Total Count | 14600 | 11800 | 4800 | 1400 | 5300 | 5650 | 8600 | 6450 |
| 8 Minute-Experiment | Alanine | 13.6 | 25.9 | 18.4 | 8.4 | 8.4 | 0.9 | 14.6 | |
| | Glutamic | 12.3 | 9.0 | 5.1 | 8.3 | 7.1 | 0.6 | 12.6 | |
| | Aspartic | 7.4 | 2.2 | 3.5 | 2.1 | 1.6 | 0.7 | 9.3 | |
| | Lactic | 49.0 | 36.3 | 24.5 | 58.3 | 45.0 | 45.8 | 29.0 | |
| | Succinic | 7.1 | 3.4 | 0(?) | 2.1 | 3.7 | 0.8 | 10.7 | |
| | Malic | 10.1 | 6.1 | 3.0 | 7.2 | 5.1 | 4.7 | 8.5 | |
| | N-EFF. | 8.5 | 15.3 | 13.6 | 7.1 | 5.2 | 2.6 | 34.9 | |
| | Recovered in Chromat | 108.0 | 98.2 | 68.1 | 93.5 | 76.1 | 56.1 | 119.2 | |
| | Undet. | (−8.0) | 1.8 | 31.9 | 6.5 | 23.9 | 48.9 | (−19.2) | |
| | Total Count | 9500 | 4170 | 3750 | 2300 | 8900 | 4550 | 1750 | |

nickel plate and counted the radioactivity by a gas-flow counter (Tracerlab. model TGC 14) after drying under infrared lamp. One microcurie of the isotope corresponded $2.25 \times 10^5$ count per minute (c.p.m.) by this counter. Each count was corrected for self absorption by weighing the plate before and after spotting the sample.

## Results

According to the time course study, the total isotope in the liver was maximum at 3 minutes after the injection of the tracer, and seemed somewhat lower at 1 and 8 minutes. This result was not consistent with the data of Busch. It may be due to the difference in amount of pyruvate injected. That is, in this experiment sodium pyruvate injected was 0.285 mg against 1 mg of Busch's experiment. In consideration of the error by the time delay for the excision of the tissue, the author adopted the 3 and 8 minute time periods as the experimental points. The incorporation of the isotope, the amount and the specific activity of each substance in each condition are presented in Table 1. The distribution of the isotope to each substance is expressed in percentage of total isotope in the tissue and presented in Table 2.

**The metabolism in carbon tetrachloride poisoning.** As an example, two chromatograms of the livers of normal and carbon tetrachloride treated rat are shown in Figure 2. As clearly seen in Figure 2 and in Table 1, the incorporation of the isotope into every fraction, especially into amino acids, was markedly decreased in

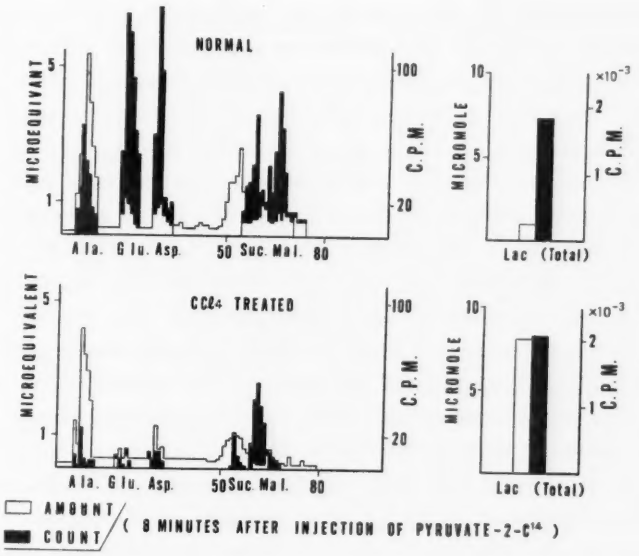


Fig. 2. Anion Exchange Chromatogram of Liver Perchloric Extract.

the liver of poisoned animal. Although the amount of each substance except lactic acid diminished, the decrease in the count exceeded it far more. So, the specific activity of each substance was also very small. The amount of lactic acid was increased markedly. But the count in it being around normal, the specific activity was considerably low (about 2/3 of normal). With reference to the per cent distribution of the isotope to each substance, more than half of the total isotepe in the tissue was found in lactic acid and very little was converted to other substances, while undetermined portion contained 40 per cent of the isotope. Count in blood may be noteworthy. In the poisoned animals, the count in blood was over twice and three times of normal at 3 and 8 minutes, respectively, after the injection of the tracer. It suggests the hindered utilization of pyruvate in the whole body in carbon tetrachloride poisoning. This experiment was repeated with smaller amount of the drug (0.05 ml/100 mg body weight), demonstrating considerable inhibition to the incorporation. Namely, the total count in the liver was 4150 c.p.m./g as compared with 9500 c.p.m./g of normal liver at 8 minute period, and the incorporation into each substance was decreased to 1/2-1/7 of normal.

**The metabolism during carcinogenesis by 3′-Me-DAB. The incorportion of the isotope into each substance:**

The results of carcinogenesis experiment are summarized in figure 3. Only 3 minute experiments are presented. As the metabolic pattern did not vary markedly between 3 and 8 minute period the data of 3 minute experiments will be treated in the following discussion. As a whole, the incorporation of the isotope into each amino acid and organic acid changed little at the early stage of 3′-Me-DAB feeding. In the late stage, however, it decreased markedly and reduced to negligible small in hepatoma. Specific activities also showed almost the same movement. The total count in the tissue was 14,600, 11,800, 4,800 and 1,400 c.p.m./g, respectively, in normal, early stage, late stage and hepatoma. The decrease in count in each substance was remarkable as follows: 2,250, 2,410, 885, 61 c.p.m./gm of tissue for alanine; 927, 685, 200, 24 for glutamic acid; 1,690, 1,470, 155, 5 for aspartic acid; 336, 330, 180, 10 for succinic acid; 656, 689, 242, 58 for malic acid in the four stages mentioned above, respectively. Lactic acid increased from the late stage, and in hepatoma it was more than 5 times of normal. On the contrary, the count in it changed as 2,322, 2,525, 1,890 and 396 respectively in the four stages. Accordingly, the specific activity was somewhat high in the early stage and very low in the late stage and hepatoma. Specific activities of amido acids were around normal in the early stage and decreased markedly in the late stage and hepatoma. Count in neutral effluent decreased in the following manner: 1,152, 735, 450 and 120. This may be due to the diminished incorporation of the isotope into glucose or other unknown compounds. Notwithstanding the data representing the strong inhitition of

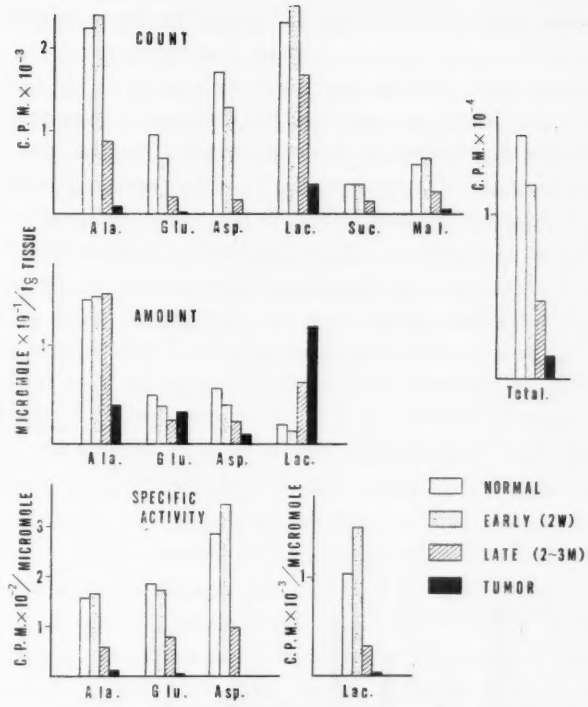


Fig. 3. Change of Pyruvate Metabolism during 3′-Me-DAB Feeding
(Three Minutes After i.v. Injection of Sodium Pyruvate-2-$C^{14}$).

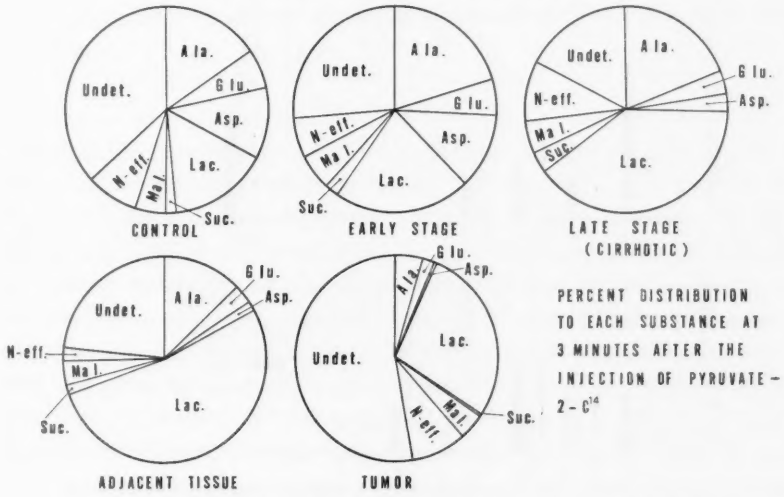


Fig. 4. Distribution of the Isotope to Each Substance at Each Stage.

pyruvate utilization in the cirrhotic liver and hepatoma, the count in blood in 3′-Me-DAB fed rats was not high. This is perhaps because pyruvate is also utilized promptly in tissues other than the liver, and the capacity is not seriously injured in the 3′-Me-DAB fed rats in contrast with the carbon tetrachloride poisoning.

**Comparison of the distribution to each substance:** To see the conversion of pyruvate to other substances, the distribution of the isotope to each substance is expressed in percentage of the total isotope in the tissue, and presented in Table 2 and Figure 4. Even in percestage, the distribution to amino acids and succinic acid was very small in hepatoma. In hepatoma, the percentage of the isotope in lactic acid was a little more than normal but less than the late stage or adjacent liver tissue at 3 minute period, and undetermined fraction contained over 50 per cent of the isotope. This part, however, was substituted by lactic acid (58.3 per cent) by 8 minutes as is seen in Table 2. This undetermined count in 3 minute period may be derived chiefly from pyruvate-2-$C^{14}$ itself. Striking difference in the distribution of the isotope was noted between hepatoma and adjacent liver tissue at 3 minute

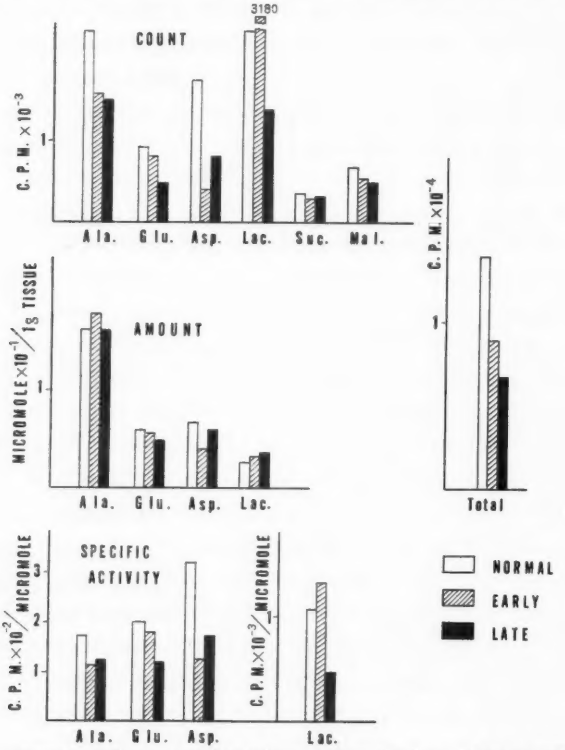


Fig. 5. Change of Pyruvate Metabolism during Choline Deficient Diet Feeding.
(Three Minutes After i.v. Injection of Sodium Pyruvate-2-$C^{14}$).

period. The patterns of distribution, however, came to resemble each other by 8 minutes. Of course the total count in hepatoma was as small as 2,300 c.p.m./g of tissue against 8,900 of the adajacent liver tissue.

**Metabolism in choline deficiency:** In comparison with the above metabolic changes, experiments of the same type were performed with two stages of choline deficient rat, as described above. The results are summarized in Figure 5. (See also Table 1 and Table 2) As shown in Figure 5, the total count decreased gradually. The incorporotion of the isotope into each substance except lactic acid decreased from early stage of 3 days. Count in lactic acid increased in the early stage and decreased in the late stage. As the amounts of these substances did not change markedly, the specific activities of them showed almost the same movement as the counts.

**The change of glycogen content:** Glycogen contents in these conditions were estimated. Normal liver, early and late stage of 3′-Me-DAB feeding, 3′-Me-DAB indeuced hepatoma, severe (0.2 ml/100 g body weight, 4 hrs.) and moderate (0.05 ml/100 g body weight, 4 hrs.) poisoning of carbon tetrachloride, early and late stage of choline deficient fatty liver, contained, respectively, an average of 5,100, 2,500, 2,400, 400, 400, 1,400, 3,700 and 4,500 mg of glycogen/100 g wet weight of tissue. That is, glycogen decreased from the early stage of 3′-Me-DAB feeding and maintain the level during cirrhosis or precancerous state, falling acutely in hepatoma to less than 1/10 of normal. In carbon tetrachloride poisoning, glycogen decreased according to the strength of the intoxication, while in choline deficiency the decrease restored to some extent in the late stage. Although the incorporation of the isotope into glycogen was also measured, it was very small even in the normal liver in this experimental condition. In the case of injured liver, the incorporation was negligible.

## Discussion

The results obtained with the 3′-Me-DAB induced hepatoma were similar to those with the transplantable tumors by Busch.[17] The conversion of pyruvate to lactate was not so tremendous in the former as in the latter. This may be due to the difference in the glycolytic activity. In any case, in hepatoma also, the inhibition of oxidative metabolism as well as the amination reaction seems to exist in situ.

In the course of carcinogenesis by 3′-Me-DAB, these changes did not occur in the early stage of 2 weeks but appeared in the late stage of cirrhosis or precancerous state, and proceeded gradually to an extremely low state of utilization in the hepatoma. This gradual decrease of the metabolic activity in the precancerous liver is consistent with the results of some enzyme assays but not others. This also corresponds with the results of Dessi[10] mentioned previously. The observation of Porter and Bruni[23] by electron microscope, that the endoplasmic reticulum swells at 48 hours

of 3′-Me-DAB feeding, while little change is found in the mitochondria even at 11 days; and the study of Allard *et al.*[24] demonstrating the decrease in the number of mitochondria in the precancerous liver cells, corroborate the present data at the cell level. These works, in accordance with this study, may indicate that the pattern of liver injupy by 3′-Me-DAB is not the direct damage of respiration or other related metabolism but the secondary one, caused by some unfavorable changes of enzymes or other factors by way of, perhaps, damaged endoplasmic reticulum. On the contrary, the pattern of injury in carbon tetrachloride poisoning or in choline deficiency seems to be the primary inhibition of respiration, since the very early diminution of pyruvate metabolism was demonstrated in both cases. This view is consistent with the recent reports of Recknagel *et al.*[25][26] and of Dianzani[27,28,29] that the DPN-dependent oxidation is first damaged by carbon tetrachloride or by choline deficiency. Although the gradual transition of metabolism during carcinogenesis was observed, acute fall at the last stage was also remarkable as seen in Figure 3. This striking difference between precancerous liver and hepatoma might suggest the existence of some essential difference in the metabolism between them. The data of Carruthers *et al.*[30] that pyridine nucleotide levels show no change during carcinogenesis but a marked decrease in the tumor, may, in part, account for these findings.

As stated in the beginning, various conditions must be taken into account to evaluate these results. First, the hindered blood supply, if it should exist, may influence the metabolism through the deficiency of oxygen and substrate——pyruvate 2-$C^{14}$ itself in this case. Indeed, Urbach and Noell[31] reported the low oxygen tension in the cutaneous cancer as compared with normal skin tissue. Hepatoma, however, is known to be relatively rich in blood supply. Cirrhrotic liver is generally considered to be irrigated by reduced portal blood and increased arterial blood, and hepatoma is supplied chiefly with arterial blood through hepatic artery. So, these marked changes during carcinogenesis may not be ascribable to the diminished blood supply. Busch also inferred this point with reference to the metabolism of other tissues,[17] ane came to the same conclusion.

Secondly, the change of cell population during carcinogenesis must be taken into consideration. According to Daoust and Cantero,[32] the parenchymal cells decreased and were replaced by the increasing bile duct cells in the course of azo dye feeding. So, the change of metabolism in liver tissue as a whole does not always represent the change of cellular metabolism. This concept, as Daoust and Cantero insist, may have to be applied also to the other experiments done with homogenetes or slices, during azo dye carcinogenesis. A correction was attempted by calculating the metabolism per cell for the decrease in parenchynral cells, according to the data of Daoust and Cantero. But the conclusion of this experiment, even in the

largest estimation of the error, need not be altered on account of the very magnitude of the change.

Finally, more work must be done to investigate the changes of metabolism during carcinogenesis. For, tnough the relationship between metabolic changes and carcinogenesis is not yet known, the possibility might exist that some metabolic defect caused by carcinogen would lead the metabolism of the cell to some deviated pattern, and finally, by affecting the nucleus through some successive reactions or some feed-back mechanisms, the cell itself to cancer.

## Summary

Changes of pyruvate metabolism during hepatocarcinogenesis by 3'-Me-DAB were investigated, using pyruvate-2-$C^{14}$ as a tracer *in vivo*. The conversion of pyruvate into lactic, succinic, malic, glutamic and aspartic acid and alanine was studied by isolating them by the anion exchange chromatography of Busch *et al.* The results were as follows:

1) Pyruvate metabolism did not show remarkable changes in the early stage of 2 weeks of 3'-Me-DAB feeding. Marked inhibition was observed in the late stage of 2-3 months, with a gradual advance to a extremely low state in hepatoma. But the difference between precancerous liver and hepatoma was so striking that the existence of some essential difference in the factors controling metabolism was suggested.

2) In explanation of these results, changes of the cellular metabolism, blood supply of the tissue and the cell population were discussed. But, in conclusion, the metabolic change of the cells is thought to be a principal factor.

3) In the carbon tetrachloride poisoned rats, strong inhibition of the pyruvate metabolism was demonstrated in the very early period of 4 hours after the intramuscular injection.

4) Chroline deficient diet caused a considerable inhibition of the pyruvate metabolism in the early stage of 3 days, and this state did not change markedly in the late stage of 30 days.

5) These results were discussed ih regard to the pattern of liver injury by these three substances.

## Acknowledgement

The author wishes to express his thanks to Prof. Tasaka and to Dr. Araki for their warm instruction and discussion, and he is also grateful to Dr. Nasuno for his helpful advice on the chromatographic technic. The author is indebted to Dr. Sugano for the examination of histological specimens. The excellent technical assistance of Miss. Furuya is gratefully acknowledged.

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[GANN, 52, 149~153; June, 1961]

# INDUCTION OF PULMONARY TUMORS IN MICE BY SUBCUTANEOUS INJECTION OF 4-NITROQUINOLINE N-OXIDE

**(Plate VIII)**

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It has been known for years that cutaneous application of carcinogenic hydrocarbons induces pulmonary tumors in mice. Murphy and Sturm (1) first demonstrated this distant action of tar upon pulmonary tissue of mice by rotating the applications of tar over different areas of the skin and thus avoiding the appearance of skin cancers. Of 40 mice painted in this manner and survived for six months, 85 per cent had multiple lung tumors. Results similar to those of Murphy and Sturm have been obtained by many investigators (2, 3), who exposed mice to tar by several routes, including subcutaneous injection and intraperitoneal injection, and elicited pulmonary tumors. Later, Andervont reported that multiple lung tumors arose in the majority of A strain mice within three months after the injection and before any of them had developed subcutaneous sarcomas, suggesting that the lung of this strain was a more delicate test object than the subcutoneous tissue. It is also evident that the susceptibility of the strains to induction of pulmonary tumors is parallel to their susceptibility to spontaneous development of pulmonary tumors. Shimkin (4) introduced more exact quantitative methods by considering the number of nodules in the lung. He also summarized the investigations concerning pulmonary neoplasms.

Very recently, a powerful carcinogenicity of 4-nitroquinoline N-oxide was found by Nakahara and his colleagues (5), and they reported that the chemical has the carcinogenic activity of the magnitude approaching that of the potent polycyclic hydrocarbons. After that, Takayama (6) succeeded to induce skin tumor by a single application of the chemical.

In the course of the present experiment, the induction of pulmonary tumors in mice was demonstrated after a single injection of 4-nitroquinoline N-oxide subcutaneously. And these results are described in this paper.

## EXPERIMENTS

Four groups of female mice of dd strain and one group of female mice of $C_{57}$bl strain were used in this experiment. They were 2 to 3 months of age, weighing 15 to 20 g, at the start of the experiment. All the mice were maintained on the mixture of rice and Oriental mouse diet (1 : 1), with an unlimited supply of water. In addi-

tion, a bit of green vegetables was given thrice a week.

The carcinogens employed were 4-nitroquinoline N-oxide and 20-methylcholanthrene. Oil solution (olive oil and cholesterol, 7 : 1) was employed as the solvent for carcinogens.

Group 1 : 20 dd strain mice received 1 mg of 4-nitroquinoline N-oxide.

Group 2 : 30 dd strain mice received 0.5 mg of 4-nitroquinoline N-oxide.

Group 3 : 12 $C_{57}$bl strain mice received 0.5 mg of 4-nitroquinoline N-oxide.

Group 4 : 25 dd strain mice received 1 mg of 20-methylcholanthrene.

Group 5 : 10 dd strain mice received 0.2 ml of the oil solution without carcinogen and served as controls.

All the mice received a single subcutaneous injection each in right axillary region. About half of the animals in group 1 died within 5 days after the injection. This fact is presumably due to the toxic effect of 4-nitroquinoline N-oxide. Then $LD_{50}$ of the chemical is seemed to be 50 mg/kg of body weight of dd mice, when injected subcutaneously. In group 2, 10 of 30 mice, died early in the course of the experiment, and only one of 12 animals died in group 3. In group 4, one-third of animals died in the course of the experiment. And in controls (group 5) only two animals died. Then, in groups 1, 3, 4 and 5, experiments were terminated at the end of the forth month, and in group 2, at the end of the third month. Animals died in the meantime were discarded.

In group 4 (20-methylcholanthrene), the development of subcutaneous tumors in the injected area was began to be detectable after three months, and half of animals had subcutaneous tumors, large or small, at the end of the experiment. While, there was no scar or growths in the injected region in mice in the other groups.

All the mice were sacrificed by cervical dislocation and autopsies made grossly. The lungs were examined for the presence and the number of white nodules and fixed in formalin. After fixation, the nodules in the lungs were counted and the number was recorded. The tissue was sectioned and stained with hematoxyline and eosin for histologic verification. Since the majority of pulmonary tumors in mice were found directly under neath the pluera, it was possible to count the tumors on the external surface of the lungs with gratifying accuracy, when they attained a fraction of a millimeter in diameter, especially after fixation (Fig. 1).

The gross appearance of the pulmonary tumors was characteristic : they were pearly, round areas, slightly raised and sharply distinct from the surrounding lung tissue. Histologically, the cells making up the tumors are fairly large, cuboidal or ovoid in shape, usually lying in a single layer on either side of a thin shred of stroma. The general pattern gives the impression that the tumor is composed of closely packed folds of an epithelial cord. Mitotic figures are present in fair number. The growths are identical in structure with those described as primary

Table 1. Pulmonary tumors in mice injected with 4-nitroquinoline N-oxide or 20-methylcholanthrene subcutaneously.

| Experimental group | Dose of carcinogen injected | Initial number of mice (strain) | Effective number of mice | Number of pulmonary tumors in mice | Tumor incidence per cent | Mean number of lung nodules per mouse |
|---|---|---|---|---|---|---|
| 1 | 1.0mg NQO* | 20 (dd) | 8 | 0, 19, 20, 21, 22, 39, 54, 54 | 87.5 | 28.6 |
| 2** | 0.5 mg NQO | 30 (dd) | 19 | 0, 3, 3, 5, 5, 7, 8. 9, 11, 14, 15, 15, 15, 15, 15, 16, 17, 21, 34 | 95.0 | 12.0 |
| 3 | 0.5 mg NQO | 12($C_{57}$bl) | 11 | 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 2 | 18.1 | 0.3 |
| 4 | 1.0 mg MCA*** | 25 (dd) | 12 | 0, 0, 0, 0, 0. 0. 0†, 0†, 0†, 2†, 2†, 25† | 25.0 | 2.4 |
| 5 (Control) | 0 | 10 (dd) | 8 | 0, 0, 0, 0, 0, 0, 0, 0 | 0 | 0 |

* NQO: 4-nitroquinoline N-oxide.

** The experiment was termined on the third month in this group only.

*** MCA: 20-methylcholanthrene.

† Mouse with subcutaneous tumor.

tumors of the lung in mice by many previous investigators. It is designated as papillary cystoadenoma (Figs. 2, 3 and 4). The induced tumors are almost invariably multiple, while spontaneous tumors are usually single.

The results of the experiment are summarized in Table 1. As is shown in the table, the final effective number consisted of 8, 19, 11, 12 and 8 in groups 1, 2, 3, 4 and 5, respectively. It is seen that, in both groups 1 and 2, lung tumors were present in most of the mice injected with 4-nitroquinoline N-oxide subcutaneously. The lung tumor incidences for groups 1 and 2 were 87.5 and 95.0 per cent, respectively. The mean number of nodules of tumors per mouse was 28.6 in group 1, while it was 12.0 in group 2. The lung tumor incidence in group 3 was 18.1 per cent, and the mean number of nodules was only 0.3. The result shows that mice of strain $C_{57}$bl are less susceptible to the induction of pulmonary tumors by subcutaneous injection of 4-nitroquinoline N-oxide. In group 4, the incidence of lung tumors was 25.0 per cent and the mean number of lung nodules per mouse was 2.4. Half of the mice in this group had subcutaneous tumors when they were sacrificed as described above. Not in a single instance in group 5, controls, was a tumor of the lung or other organ encountered.

From these results, it was found that lung tumors were evoked in the both strains of mice, dd and $C_{57}$bl, with 4-nitroquinoline N-oxide, when the chemical was introduced by the subcutaneous injection, without showing any lesions suggesting cancer in the injected region. The results also demonstrated that number of tumor nodules induced in the lungs with the chemical is more numerous in mice injected with

large amount of the agent than in mice with small dose. Mice of strain $C_{57}$bl are less susceptible to the induction of lung tumors with 4-nitroquinoline N-oxide than mice of dd strain. And lung tumors of dd strain mice are more easily induced when the injection of 4-nitroquinoline N-oxide was made subcutaneously than in the case of 20-methylcholanthrene.

## Discussion

Spontaneous tumors of the lung are uncommon in certain strains of mice. In dd strain it is rare to find a lung tumor in animals 7 months old or younger as previously reported (7, 8). With these fact before us we have attempted to eliminate as completely as possible the chance of spontaneous tumors from our experimental materials by killing all the mice before they were 7 months old.

On the other hand, it has been founded by many investigators that mice failing to develop skin cancer as the result of application of carcinogenic hydrocarbons have tumors of the lungs, as described above. In this experiment, it was found that pulmonary tumors are induced by a single subcutaneous injection of 4-nitroquinoline N-oxide, the dose of the agent being directly proportional to the number of tumors per animal. And the susceptibility of the strains to induction of pulmonary tumor was parallel to spontaneous development of pulmonary tumors. Recently, Nakahara and Fukuoka (9) reported that the carcinogenicity of 4-nitroquinoline N-oxide is less than that of 20-methylcholanthrene when applied externally. In contrast to this the incidence of pulmonary tumors in mice which received 4-nitroquinoline N-oxide is earlier and higher than in mice which received 20-methylcholanthrene. While the development of subcutaneous tumor in the region of injection appeared as early as at the third month in the case of 20-methylcholanthrene, no such growth was seen even at the end of the experiment in mice which received 4-nitroquinoline N-oxide.

The difference in the mode of action between 4-nitroquinoline N-oxide and 20-methylcholanthrene is not clear at present. One possibility is that 4-nitroquinoline N-oxide might be absorbed or dispersed soon after the application, and would get into lungs through the lymphatics. The possibility of the inhalation of the carcinogen can be eliminated in this case.

The most difficult point of interpretation in this study is the type of cell giving rise to the tumors. That it is usually derived from the alveoli is certain in the majority of preparations, as many previous investigators observed in the cases of urethane and other chemicals (10–14). However, it is noteworthy that hyperplastic findings were frequently observed in epithelia of a bronchus or bronchioles of the lungs in mice which received 4-nitroquinoline N-oxide.

## Summary

Multiple pulmonary tumors in mice of dd or $C_{57}$bl strains were induced by a single injection of suitable quantities of 4-nitroquinoline N-oxide subcutaneously. And mice of strain dd are more susceptible than mice of $C_{57}$bl to the induction of pulmonary tumors. The observations recorded in this paper indicate that in dd strain mice the lung response occurred in a relatively short period of time, i.e., as early as three months. The nodules of tumors in the lungs per mouse are more numerous when the dose of the agent injected is large than when it was small. Induction of pulmonary tumors in dd mice by the injection of 4-nitroquinoline N-oxide is more conclusive than by the injection of 20-methylcholanthrene.

## Explanation of Plate VIII

Fig. 1. Multiple induced tumors of the lung of mouse which received 1 mg of 4-nitroquinoline N-oxide injection subcutaneously (Fourth month).

Fig. 2. Microscopic picture of a tumor of Fig. 1.

Fig. 3. Higher magnification of Fig. 2.

Fig. 4. A fully developed tumor.

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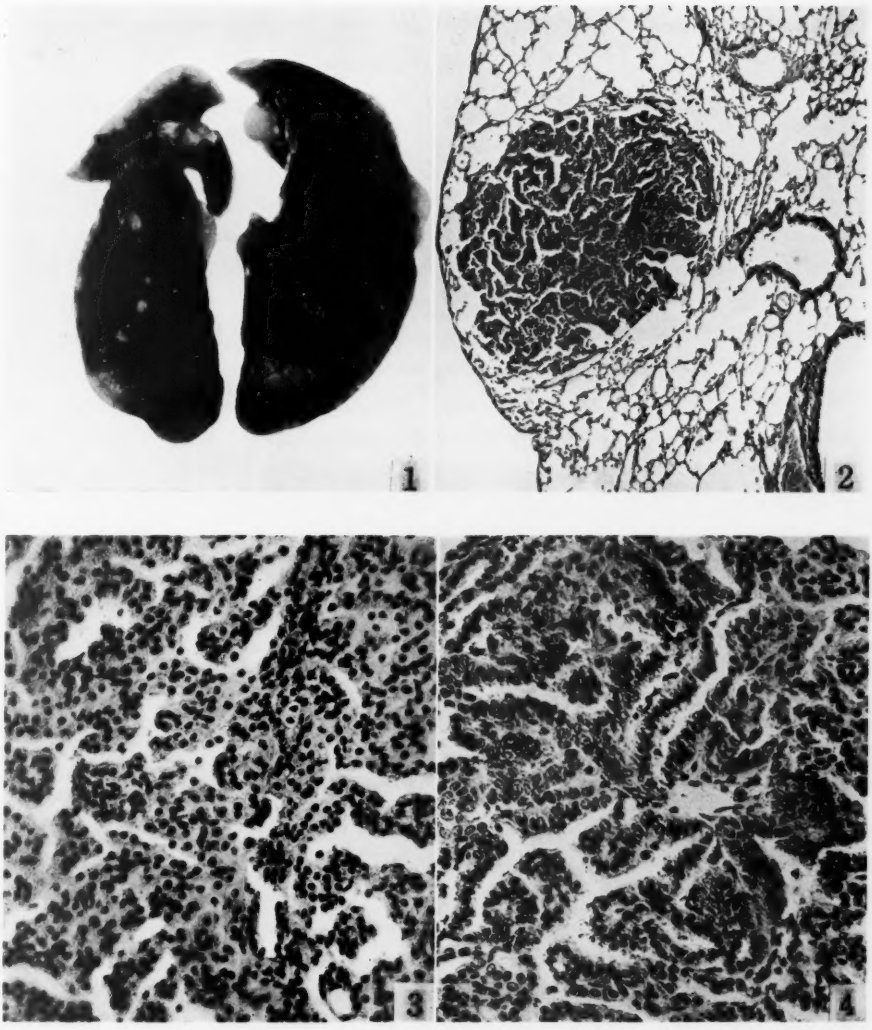
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[GANN, 52, 155~164; June, 1961]

# ON THE RELATIONSHIP BETWEEN CONGO RED INDEX AND QUANTITATIVE HISTOLOGY IN THE RAT LIVER DURING AZO DYE CARCINOGENESIS

(Plates IX and X)

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Previously, several workers (1-9) observed the exsistence of close relationship between the functional level of the reticulo-endothelial system (RES) and the growth of tumor.

In our previous report, Ito et al. (10-13) suggested that the hepatic reticulo-endothelial cells (Kupffer cells) might play an important role in the destruction of p-dimethylaminoazobenzene (DAB) in the rat liver. During carcinogenesis by DAB, the histological pattern of the liver is known to be markedly altered at the various degrees according to the stages of experiments. For instance, Daust and Striebich et al. (14-16) have observed changes in the numerical population of the various cell types presented in the livers of normal rats and rats fed with DAB.

The most commonly used functional test of RES is that based on congo red index published by Adler-Reimann (17), and our present investigations are based on Yasuoka's modification of the Adler-Reimann method (18). In these investigations, the authors also studied the relationship between the congo red index and the quantitative histology, especially hepatic reticulo-endothelial cells, of the rat liver during carcinogenesis caused by various azo dyes.

## Materials and Methods

In the first series of experiments, 210 adult male albino rats (Wistar strain, 180-270 g) were used. Each congo red index of those rats was determined by Yasuoka's modified Adler-Reimann method (18). The test was based on the following principles: 0.2 cc of 1 per cent watery congo red solution per 100 g body weight was injected into *V. femoralis*, and three and sixty minutes, respectively, after each injection, blood samples were withdrown and congo red concentration in the serum or plasma was colorimetrically determined. The light absorption data were obtained by Beckmann-type spectrophotometer of approximately 0.03 mm slight-width and 10 mm wide corex cells. After light centrifugation, the optical density of the solution was determined at 500 m$\mu$ in the spectrophotometer.

The significance of congo red index was as follows:

$$\text{Congo Red Index} = \frac{\text{Congo red concent. in plasma (60 min.)}}{\text{Congo red concent. in plasma (3 min.)}} \times 100$$

Then, rats whose congo red index showed under 20 or over 40 were all excluded from the total number.

In the second series of experiments, which were started one week after the first series, 95 rats that had been selected a week before by the above method were used. They were divided into four groups including the control group. Those in the first group were fed with semi-synthetic diet containing 3′-methyl-p-dimethylaminoazobenzene (3′-Me-DAB) at the concentration of 0.06 per cent. The second group received semi-synthetic diet containing 0.1 per cent o-aminoazotoluene (OAT). The third group received semi-synthetic diet containing 0.1 per cent methyl-orange (M-Orange). The rats of the 4 th group were fed with semi-synthetic basal diet without azo dye. The basal diet was patterned after that of Price et al. (19), except that an antibiotic was added (Table 1.). These rats were also given water *ad libitum*.

Table 1. Ingredients of basal diet (in g/kg diet)

| | |
|---|---|
| Rice powder | 750.0 |
| Casein | 100.0 |
| Corn oil | 100.0 |
| Salt mixture (Osborne-Mendel) | 40.0 |
| Thiamin chloride | 0.003 |
| Riboflavin | 0.0015 |
| Pyridoxine hydrochloride | 0.003 |
| Cholin chrolide | 0.030 |
| Calcium pantothenate | 0.007 |
| Nicotinic acid | 0.005 |
| Cod liver oil | 0.300 |
| Tetracycline hydrochloride | 0.100 |

Certain weeks (2, 4, 6, 8 and 10 weeks) after initiation of the diet, each congo red index was once more determined by the above mentioned method. Then, the average differences in the values of congo red index between the pre-treated stages (first series) and post-treated stages (second series) were determined at various weeks among each individual group of 4 to 5 rats.

After that, they were sacrificed by decapitation. The specimens of the liver in each rat was fixed in 10 per cent formalin and Susa solution. Histological sections (4$\mu$ thick) were prepared by the routine procedure and stained with hematoxylin-eosin and periodic acid-Schiff (PAS).

For the differential counting of cell types in the liver specimens, a reticule was placed in one of the oculars. Fields were set at random on a liver section, and all the nuclei included in these fields were examined under oil immersion. The counting was limited to the following cell types: parenchymal cells, so-colled ' littoral cells ' (14, 16, 20), bile duct cells, cells of connective tissue, and cells of blood vessel wall. At least one thousand nuclei per field were taken at random per rat. Blood cells were not included in the counting.

At the same time, the avarage percentage of PAS-positive cells within two-hundred of the reticulo-endothelial cells in the above rat liver specimens were determined under oil immersion.

## Results

The frequency distribution of variation in congo red index of total rats in the first series of experiments was given in chart 1.

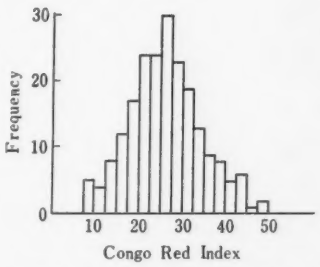


Chart 1. Frequency distribution of variation in congo red index of the total of 210 adult male albino rats.

Then, the difference in the values of congo red index was determined in each rat between the pre-treated stage and the post-treated stage and was presented under the various conditions in Tables 2, 3, 4, 5 and Chart 2. In the rats fed with 3′-Me-DAB containing diet, the differences in the values of congo red index was highest compared with the corresponding values in other groups. That of 3′-Me-DAB supplemented group gradually rose during the experiment. The similar tendency was noticed in OAT supplemented group, but it was lower than in 3′-Me-DAB supplement group. In the rats with M-Orange diet, however, the difference was lowest at the end of second week but afterwards it gradually approched the control level, and then after four weeks it slowly rose again. The difference at the end of 10 th week, however, was lowest except in the control. In other words, the difference of functional activity in RES was weakest in the rats fed with M-Orange diet, excepting the control.

Changes in the cell population in the rat livers that had been determined by Daust's method (14), were shown in Table 6 and Charts 3, 4, 5, and 6. The number of paren-

Table 2. Congo red index in the rats fed with 3′-Me-DAB containing diet.

| Animal No. | Experimental Periods (Weeks) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | | 4 | | 6 | | 8 | | 10 | |
| | I | II | I | II | I | II | I | II | I | II |
| 1 | 36.7 | 55.6 | 20.0 | 49.1 | 32.0 | 47.5 | 22.6 | 40.8 | 22.9 | 33.8 |
| 2 | 21.3 | 24.4 | 22.1 | 38.3 | 24.3 | 42.0 | 28.5 | 42.7 | 23.6 | 48.9 |
| 3 | 35.2 | 43.6 | 25.6 | 40.8 | 33.2 | 48.6 | 30.7 | 48.6 | 28.9 | 38.5 |
| 4 | 21.1 | 35.0 | 25.9 | 26.4 | 28.7 | 45.0 | 20.5 | 44.1 | 20.9 | 42.2 |
| 5 | 23.2 | 27.1 | 21.5 | 37.4 | 31.3 | 45.3 | 25.0 | 35.9 | 26.3 | 52.5 |
| Average of Diff. C.I. | +9.7 | | +15.4 | | +15.8 | | +17.0 | | +18.7 | |

I: Pre-treated stage II: Post-treated stage

Table 3. Congo red index in the rats fed with OAT containing diet.

| Animal No. | Experimental Periods (Weeks) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | | 4 | | 6 | | 8 | | 10 | |
| | I | II | I | II | I | II | I | II | I | II |
| 1 | 27.0 | 30.3 | 35.1 | 36.9 | 30.1 | 32.7 | 21.9 | 37.9 | 26.2 | 41.3 |
| 2 | 28.6 | 34.2 | 23.2 | 38.8 | 20.4 | 33.3 | 24.6 | 37.1 | 29.1 | 39.6 |
| 3 | 22.4 | 25.0 | 22.7 | 28.9 | 26.2 | 29.3 | 25.2 | 35.1 | 27.8 | 35.6 |
| 4 | 33.5 | 27.4 | 23.0 | 31.6 | 20.7 | 39.1 | 27.9 | 40.6 | 25.5 | 29.2 |
| 5 | 28.7 | 28.6 | — | — | 31.1 | 27.7 | 32.2 | 40.2 | 20.9 | 34.9 |
| Average of Diff. C.I. | +1.1 | | +8.1 | | +6.7 | | +11.8 | | +10.2 | |

I: Pre-treated stage II: Post-treated stage

Table 4. Congo red index in the rats fed with M-Orange containing diet.

| Animal No. | Experimental Periods (Weeks) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | | 4 | | 6 | | 8 | | 10 | |
| | I | II | I | II | I | II | I | II | I | II |
| 1 | 34.9 | 24.7 | 18.8 | 28.7 | 33.6 | 38.6 | 30.9 | 41.1 | 20.1 | 31.6 |
| 2 | 31.9 | 26.1 | 26.6 | 33.1 | 36.8 | 46.3 | 25.2 | 33.4 | 33.1 | 34.3 |
| 3 | 26.5 | 26.4 | 22.0 | 28.5 | 27.2 | 31.7 | 22.7 | 34.2 | 21.9 | 38.3 |
| 4 | 28.9 | 21.5 | 26.1 | 31.4 | 20.0 | 26.2 | 30.4 | 39.5 | 24.9 | 32.9 |
| 5 | 31.3 | 28.5 | 44.7 | 38.1 | — | — | 26.4 | 25.0 | — | — |
| Average of Diff. C.I. | −5.1 | | +4.3 | | +6.4 | | +7.5 | | +9.3 | |

I: Pre-treated stage II: Post-treated stage

Table 5. Congo red index in the rats fed with basal diet without azo dye.

| Animal No. | Experimental Periods (Weeks) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | | 4 | | 6 | | 8 | | 10 | |
| | I | II | I | II | I | II | I | II | I | II |
| 1 | 24.5 | 37.6 | 26.5 | 24.0 | 25.0 | 34.8 | 23.1 | 35.3 | 27.8 | 34.8 |
| 2 | 37.3 | 29.6 | 19.6 | 20.2 | 26.2 | 20.2 | 28.8 | 33.3 | 38.7 | 37.3 |
| 3 | 29.2 | 30.1 | 28.1 | 26.0 | 28.0 | 31.5 | 21.7 | 23.2 | 27.6 | 37.7 |
| 4 | 27.5 | 39.3 | 24.0 | 20.5 | 20.0 | 23.3 | 29.9 | 31.8 | 34.7 | 44.0 |
| 5 | 24.6 | 31.6 | 21.6 | 27.3 | — | — | — | — | 31.6 | 34.3 |
| Average of Diff. C.I. | +3.0 | | −2.8 | | +2.7 | | +5.0 | | +4.4 | |

I: Pre-treated stage II: Post-treated stage

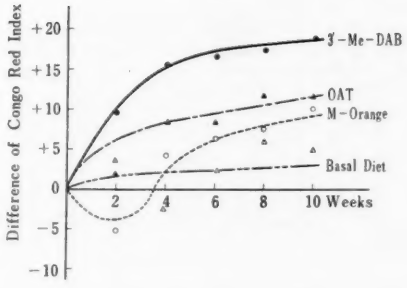


Chart 2. Comparison curves derived from the differences in the values of each other between the pre-treated stage and the post-treated stage for rats fed with the various diets.

Table 6. The average percentages of the cell population in the rat livers of the various groups.

| Weeks | Cell types | Experimental Groups | | | |
|---|---|---|---|---|---|
| | | 3'-Me-DAB | OAT | M-Orange | Basal Diet |
| II | Parenchymal C. | 64.86 | 68.16 | 68.59 | 69.38 |
| | Littoral C. | 26.52 | 28.80 | 22.89 | 27.16 |
| | Conn. T. El. C. | 8.73 | 3.04 | 8.85 | 3.48 |
| IV | Parenchymal C. | 67.30 | 64.17 | 65.79 | 69.58 |
| | Littoral C. | 15.87 | 31.03 | 25.69 | 28.05 |
| | Conn. T. El. C. | 6.82 | 4.30 | 8.56 | 3.17 |
| VI | Parenchymal C. | 59.13 | 65.71 | 69.17 | 69.76 |
| | Littoral C. | 17.35 | 29.13 | 24.67 | 26.33 |
| | Cann. T. El. C. | 17.85 | 5.16 | 6.23 | 3.90 |
| VIII | Parenchymal C | 54.23 | 64.61 | 66.06 | 65.99 |
| | Littoral C. | 27.08 | 29.28 | 25.90 | 30.36 |
| | Conn. T. El. C. | 18.70 | 6.12 | 8.03 | 3.67 |
| X | Parenchymal C. | 46.42 | 61.48 | 64.46 | 63.84 |
| | Littoral C. | 27.73 | 29.41 | 31.03 | 31.07 |
| | Conn. T. El. C. | 25.55 | 9.10 | 4.30 | 2.30 |

chymal cells in the liver specimens of the rats fed with basal diet was 70 per cent of the total number, and that of the littoral cells was 25 per cent, leaving 5 per cent that consists of the other cell types. Changes in the cytological composition of the liver in basal diet group were not remarkable thoroughout the experimental stages. Changes in the cytological composition of the rats fed with M-Orange were similar to those of basal diet group. In the OAT supplemented group, the percentage of increase in the number of littoral cells was highest. The connective tissue elemets, however, including the bile duct cells, cells of the connective tissue, and cells of the blood vessel wall, increased but slightly in number in 10 weeks. The cell population

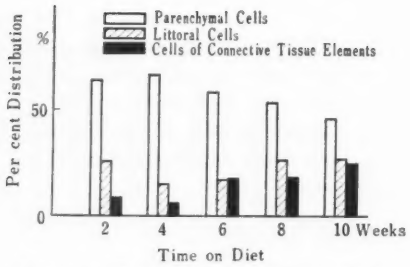


Chart 3. Changes in the cell population in the liver specimens of the rats fed with 3′-Me-DAB containing diet.

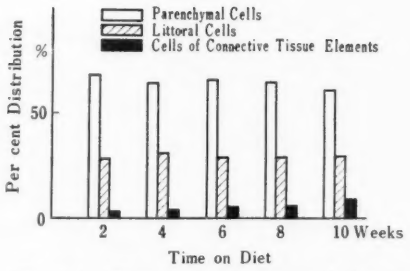


Chart 4. Changes in the cell population in the liver specimens of the rats fed with OAT containing diet.

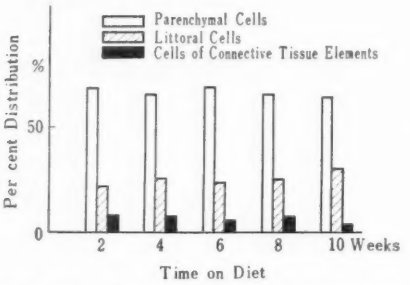


Chart 5. Changes in the cell population in the liver specimens of the rats fed with M-Orange containing diet.

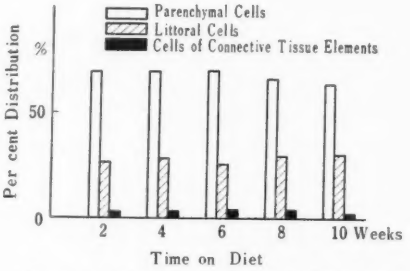


Chart 6. Changes in the cell population in the liver specimens of the rats fed with basal diet without azo dye.

of the rat livers in the present experiments showed striking change in rats fed with 3′-Me-DAB, due mainly to an extensive population of connective tissue elements. Changes in the percentage of littoral cells were various. The ratio of the littoral cells was 26 per cent of the whole, and was lowest at the end of the 4 th week, but it gradually increased toward the end of the 8 th week and the 10 th week. Furthermore, the parenchymal cells of 3′-Me-DAB fed rats gradually decreased in number, and at the end of the 10 th week they were 46 per cent of the whole. In our present study, no animal was observed for longer than 10 weeks, and therefore, no tumor was observed. Microscopically, at the end of the 10 th week, the proliferation of connective tissue elements was noticed in the rats fed with 3′-Me-DAB. Then a slight proliferation of connective tissue cells was recognized in OAT supplemented group. However, in the other groups, histological patterns were not so remarkablly changed. There were no degenerative changes in the parenchymal cells of the basal diet group during the whole course of the experiments (Figs. 1, 2, 3 and 4).

The average percentages of PAS-positive reticulo-endothelial cells in the rats of

Table 7. The average percentages of PAS-positive cells in the rat livers of the various groups.

| Weeks | Experimental Groups | | | |
|---|---|---|---|---|
| | 3′-Me-DAB | OAT | M-Orange | Basal Diet |
| II | 12.8 | 17.1 | 34.7 | 22.7 |
| IV | 15.7 | 26.5 | 49.9 | 22.8 |
| VI | 10.6 | 13.0 | 21.4 | 22.7 |
| VIII | 9.6 | 15.8 | 13.7 | 21.0 |
| X | 10.5 | 14.4 | 24.8 | 26.0 |

each group were shown in Table 7. In the 3′-Me-DAB supplemented group, the average percentages of PAS-positive cells were gradually decreased toward the end of the 10th week, while, those of the PAS-positive cells were lower than in the other groups. In the M-Orange supplemented group, excepting the control, the average percentages of PAS-positive cells in the rat livers were found to be the highest (Fig. 5). However, the percentages of PAS-positive cells in each group tended to decrease toward the end of the experiments (Fig. 6). In the control group, PAS-positive cells were 21 to 26 per cent, which showed that the differences in the percentages were not so remarkable during the whole course of the experiments.

## Discussion

Various methods of tests on the functional activity of RES have been reported by many workers (17-18, 21-24), but the most commonly used has been Adler-Reimann's congo red method (17) expressed in terms of congo red index. A low congo red index indicates rapid absorption of the dyes.

In the present investigations, it has been observed that the congo red index maintained its high level to the last stage of the experiments in groups supplemented with azo dyes. Especeally in 3′-Me-DAB supplemented group, the functional activity of RES was lowest in our present experiments. Furthermore, in the M-Orange supplemented group, it has been observed that the decrease in the functional activity of RES was slight compared with the groups supplemented with the other dyes. These results may suggest that the various azo dyes attack the RES. On the other hand, Miller et al. (25-27) have reported on the carcinogenicity of certain azo dyes in the rats. In their investigations, 3′-Me-DAB was proved to be more powerful than OAT, but M-Orange has not been proved to have any carcinogenicity in rats. From these and from our present results, an interesting relationship may be observed between the carcinogenicity of azo dyes in the rats and the grade of disturbances in the functional activity of RES determined by the congo red index. The azo dyes which had high potency as hepato-carcinogen was proved to give severe disturbances

on the functional activity of RES. Recently, there have been observations (1, 5) on the functional activity of RES in cancer patients or in rabbits that received prolonged administration of dibenzanthracene. Furthermore, it is assumed that the RES may play an important role in carcinogenesis. Jaffé (23) discussed that the functional activity in the liver reticulo-endothelial cells could not be observed in liver parenchymal cells and Kupffer cells separately both the cells making a single functional unit. The present results suggest that these azo dyes attack not only Kupffer cells but also liver parenchymal cells.

Herpelan et al. (21, 24) reported that the phagocytic activity, which is the most important part of the functional activity of RES, was most remarkable in the hepatic reticulo-endothelial cells. Azuma et al. (28) observed the presence of analogous tendency between the congo red index of the entire body of a rat and that of the perfused liver of the same rat. Our present investigations made along this line through the rat liver specimens by routine examination and numerical population of the various cell types showed that the numerical population of cell types in the liver during carcinogenesis by DAB was essentially the same with that described by Daust et al. (14–16). It was concluded, therefore, that there was no relationship between the congo red index and the numerical population of various cell types in a rat liver.

Teilum (29) and Akazaki (30) reported that in response to unspecific stimuli given on the reticulo-endothelial cells marked amount of globular or finally granular PAS-positive substance appeared in the cytoplasm. In our present study, the average percentages of PAS-positive cells were found to be low in 3′-Me-DAB supplemented group in which high values of congo red index were recognized. The results indicated the exsistence of some relationship between the congo red index and the average percentages of PAS-positive reticulo-endothelial cells. Microscopically, the findings of liver specimens within a given group were quite similar during the whole course of experiments to those reported by several previous observers (19, 31–33).

The cardinal role of the RES in immunity has been suggested by a number of investigators (34). It had also been claimed that the gamma-globulin-producing cell system in RES inhibited antibody production (35). More recently, Winzler and several authorities (36–38) have reviewed that the levels of alpha-1-, alpha-2-, and beta-globulin in blood might increase significantly in neoplastic diseases, wheras those of gamma-globulin did not show any conspicious change. Our present study suggests that these changes in the levels of globulin in blood during azo dye carcinogenesis are due to the dysfunction of the RES.

As the results of our present investigations it has been found that the dysfunction of RES was remarkable in the group supplemented with high carcinogenic substances, but it was obscure in the low or non-carcinogenic substances. Therefore, in

3'-Me-DAB supplemented group there was significant correlation between the levels of the congo red index and the carcinogenic activity of the rat liver.

Ito et al. (10-13) and Azuma et al. (28) previously reported that the DAB-destroying activity of the rat liver was increased by stimulation of the hepatic reticulo-endothelial cells. In the initial stage of carcinogenesis by azo dyes, the destruction of azo dyes in the hepatic tissue was slight when the carcinogenic activity was high, but it was great when there was low or no carcinogenic activity. Therefore, the concentration of azo dyes in the hepatic tissue was remarkable in the high potency carcinogen treated rats. From this correlation, it seems reasonable to suggest a tenteative hypotesis that the high concentration of azo dyes in the hepatic tissue may offer some favorable conditions to the carcinogenesis of azo dyes in rats.

## Summary

1. The studies described in this paper were concerned with the relationship between the congo red index and quantitative histology in the rat liver during carcinogenesis by azo dyes, including 3'-Me-DAB, OAT, and M-Orange. Functional test of RES was based on Yasuoka's modified Adler-Reimann method. The histological patterns of the liver were based on the routine examination and Daust's method.

2. The azo dye which had high potency as hepato-carcinogen gave severe disturbances on the functional activity of RES. But it was obscure in the low or non-carcinogenic substances.

3. Histological findings were similar to those reported by various observers. However, the results indicated the exsistence of some relationship between the congo red index and the average percentages of PAS-positive reticulo-endothelial cells.

4. It was concluded that the relationship between the congo red index and the numerical population of various cell types in the rat liver was not remarkable.

Acknowledgments

The authors wish to thank Prof. H. Sato, Department of Pathology, School of Medicine, Nagoya City University, for valuable discussions on this work and for the many technical advices by Dr. Y. Sayama, and Dr. M. Yasuoka.

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## Explanation of Plates IX and X

Fig. 1. The liver of a rat treated with 3′-Me-DAB containing diet for 10 weeks. Marked proliferation of connective tissue elements and hypertrophic changes in the parenchymal cells. Hematoxylin and eosin; ×100.

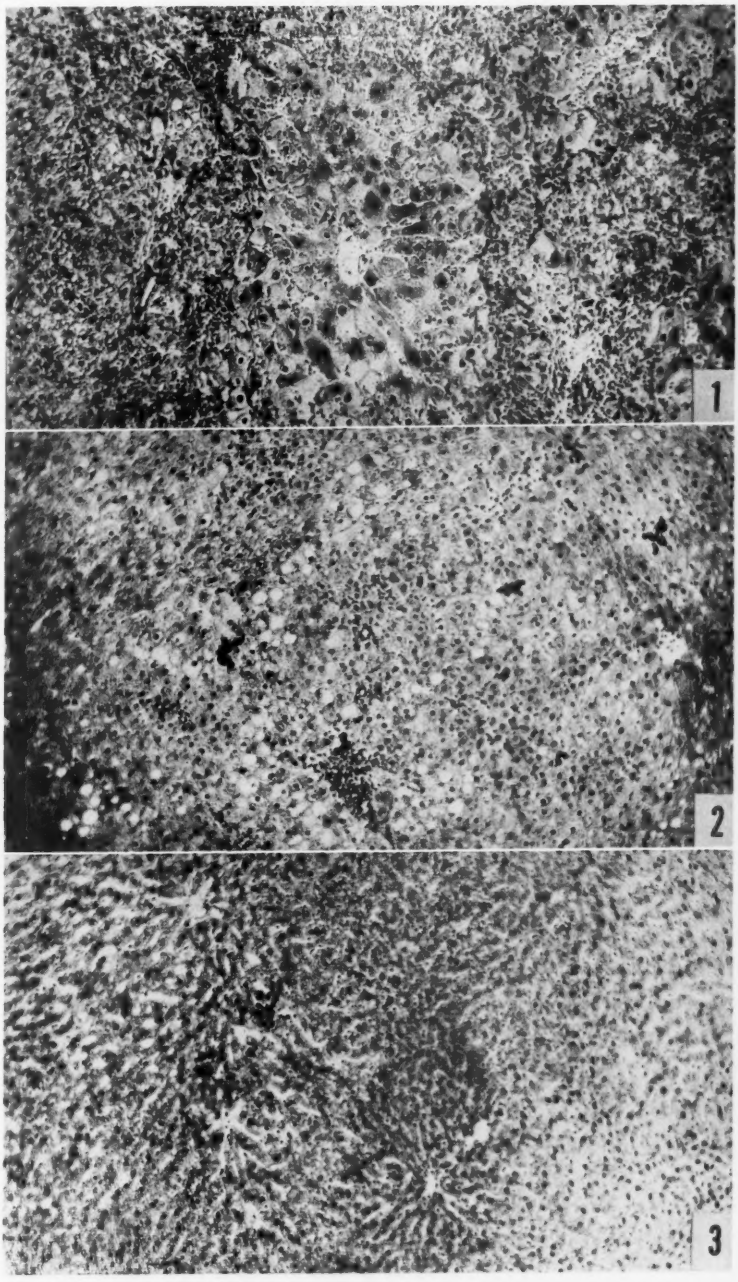
Fig. 2. The liver of a rat treated with OAT containing diet for 10 weeks. Slight proliferation of connective tissue elements and inflammatory cell infiltration into the stroma. Hematoxylin and eosin; ×100.

Fig. 3. The liver of a rat treated with M-Orange containing diet for 10 weeks. No remarkable changes in the liver parenchymal cells. Hematoxylin and eosin; ×100.

Fig. 4. Microphotograph of a rat liver of the control group in the same stage. Hematoxylin and eosin; ×100.

Fig. 5. Marked PAS-positive cells in the liver of a rat treated with M-Orange containing diet for 2 weeks. PAS stain; ×400.

Fig. 6. Few PAS-positive cells in the liver of a rat treated with 3′-Me-DAB containing diet for 2 weeks. PAS stain; ×400.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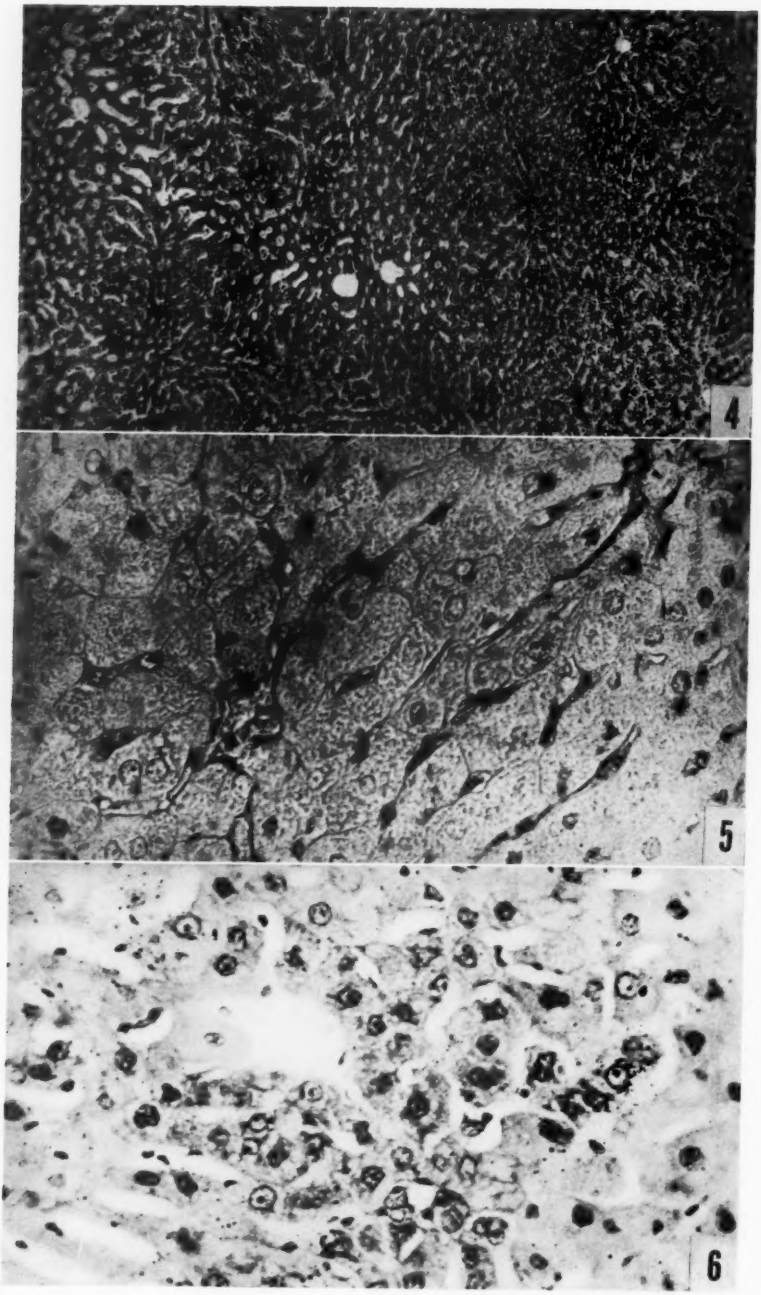
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[GANN, 52, 165~171; June, 1961]

# EFFECT OF 4-NITROQUINOLINE N-OXIDE PAINTING ON AZO DYE HEPATOCARCINOGENESIS IN RATS, WITH NOTE ON INDUCTION OF SKIN FIBROSARCOMA

**(With Plates XI and XII)**

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## INTRODUCTION

Many factors are known to cause an augmentation of the production of azo dye hepatic carcinoma. In 1943 Muta[6] reported the significant fact that hepatic carcinoma can be produced in rat and mouse which were given a short o-aminoazotoluene feeding, inadequate to produce liver tumor, if they were painted later with solutions of arsenic trioxide, 3 : 4-benzpyrene and coal tar on the skin. On the other hand, Glinos and his co-workers[3] observed that partial hepatectomy was relatively effective in accelerating the rate of appearance of hepatic carcinoma in rat liver due to p-dimethylaminoazobenzene (DAB) feeding. Quite recently Muta's experiment above mentioned was further confirmed by Odashima[9]. In his experiment, the rate of liver cancer formation was increased in the group which was fed with DAB diet only up to the stage of the formation of adenomatous nodule and then followed by 20-methylcholanthrene (MC) painting on normal diet.

4-Nitroquinoline N-oxide (4-NQO), whose powerful carcinogenicity was recently reported by Nakahara and his colleagues[7], reacts promptly in biological milieu[1,4], and the reaction products of this substance have been shown to be largely without carcinogenicity[8]. The simplicity of chemical structure with only a single reactive center, that is, the nitro radical at position 4 in association with the polar N-oxide group, makes this molcule almost an ideal substance in the study of carcinogenic mechanism.

The present study was attempted in order to observe the possible remote influence of the cutaneous administration of 4-NQO on rat liver which was carcinogenically prepared by preliminary feeding with DAB at an inadequate level for the actual production of liver cancer.

## MATERIAL AND METHODS

**Animals:** Experiment was started with 131 albino rats, 116 females and 15 males, of the average body weight of around 110 g.

**DAB diet:** DAB dissolved in olive oil was evenly mixed with the basal diet* at the rate of 0.6 g per kg and each rat was given 10 g of the DAB diet a day approximate total amount of DAB ingested being calculated from the total amount of the diet consumed through the experimental period.

**Painting procedure:** 0.5% 4-NQO and 0.3% MC, both dissolved in acetone, were applied with a capillary pipette to the skin at the central ares of the back, twice a week, about 1/20 cc at each application. The hair was clipped off 1 or 2 days prior to the initial treatment.

**Experimental group:** The animals were divided into five groups according to the experimental design as follows:

Group 1. Fed with the DAB diet for 3 months, and then continued on basal diet only.

Group 2. Fed with the DAB diet for 3 months. After which period received MC painting for following 6 months on basal diet.

Group 3. Fed with the DAB diet for 3 months, then received 4-NQO painting for the following 6 months on basal diet.

Group 4. Received 4-NQO painting for first 6 months, on the basal diet, and then fed with DAB diet for 3 months without 4-NQO painting.

Group 5. Received 4-NQO painting for 6 months, and fed with basal diet throughout.

At the end of DAB feeding one or two rats were sacrificed and liver changes were examined histologically.

All the animals dying after the 160th experimental day were autopsied and findings noted. Experiment was terminated on the 420th day by killing all the survivors. Tissues were fixed in 4% formalin solution, and paraffin sections were stained with hematoxylin-eosin. Ten or more blocks of the liver were histologically examinad from each animal.

## Results

**Histological findings of the liver immediately after the 3 months' DAB feeding.**

In the 3 cases out of the 4, newly formed bile ducts were abundantly observed accompanied by proliferation of connective tissue, especially in the periportal area. Cholangiofibrosis of moderate degree was seen in one case. Slight proliferative change of hepatic cells was found in 3 cases. There was, however, neither adenomatous hyperplasia nor hepatoma developing in any of the animals.

**Histological findings on the liver of animals sacrificed or died after 160th day.**

*Composition of basal diet: 52% of crude carbohydrate, 24.8% of crude protein, 7% of water, 5.7% of ash, 5.6% of crude fat and 4.7% of cellulose.

*Group 1.* (Three months' DAB feeding): Seventeen rats died of pneumonia between the 160th and 420th day. The remeining 6 rats were sacrificed on the 420 th day. Histological examination of the liver revealed conspicuous dilatation of both central veins and the intralobular blood sinuses in all cases. Marked cholangiofibrosis was found in 2 cases. Cyst formation was found in 7 cases, 2 of which showed multiple cysts but without any proliferation of lining epithelial cells. No liver cancer was found in this group.

*Group 2.* (Three months' DAB feeding followed by 6 months' MC painting): Eleven rats died between the 160th and 420th day. Moderate adenomatous hyperplasia was found in one case which died on the 420th day. Eight survivors were all sacrificed on the 420th day, among which one animal showed primary liver cancer without metastasis, which was histologically diagnosed as trabecular hepatoma. Marked adenomatous hyperplasia was found in 2 other cases, while extensive cholangiofibrosis was observed in 3, in one of which the change was so severe that the 3rd and 4th lobes were entirely replaced by firm, irregularly shaped masses. Histologically, there was marked proliferation of the blie ducts associated with considerable fibrosis. Hyalinous change of the stroma was also observed.

*Group 3.* (Three months' DAB feeding followed by 6 months' 4-NQO painting): In total, 23 rats died between the 160th and 420th day, 4 of which showed development of liver cancer. Four rats out of these 23 died with fibrosarcoma which developed at the painted areas, and the others of pneumonia. Among the 3 survivors sacrificed on the 420th day, 2 were found bearing primary cancer of the liver. Proliferation of newly formed bile ducts was observed in 14 cases, and 9 of which showed characteristic picture of cholangiofibrosis. Cyst formation of various degree was found in 14 cases.

The case of hepatic carcinoma occurring in this group may be described as follows:

Case 1: This animal died on the 160th day due to liver cancer. The second lobe of the liver was almost entirely occupied by two large tumor nodules involving partly the 3rd lobe. A few metastases were found in the mesenteric lymph-nodes. Histologically, this tumor was diagnosed as hepatoma with tubuloglandular pattern (Fig. 1 and 2).

Case 2: This rat was killed at the 210th experimental day, the tumor of which was definitely palpated. The 2nd, 3rd and 4th lobes of the liver were replaced by white irregularly shaped tumor masses. In the 5th lobe, an ovoid tumor mass measuring $3.0 \times 3.2 \times 2.0$ cm with central cyst formation was found. Histologically, this was composed of two different types of tumor, one a typical trabecular hepatoma with slight tendency of forming glandular structures, and the other solid hepatoma of large cellular type. Moreover, extensive cholangiofibrosis was also observed.

Case 3: This rat died on the 360th day due to extensively metastaizing fibrosarcoma which developed in the area of 4-NQO painting. One white nodule measuring 0.5×0.3 cm found in the lower part of the 3rd lobe showed characteristic picture of trabecular hapatoma histologically. The other parts of the liver were remarkable with the presence of small foci of proliferating hepatic cells surrounded by connective tissue and newly formed bile ducts. Marked cystic dilatation of the bile ducts at the periportal area was also noticeable.

Case 4: This rat died on the 398th day. The 2nd and 5th lobes of the liver were entirely occupied by rather soft, ovoid tumor masses measuring 3.5×2.5×2.0 cm, respectively (Fig. 3 and 4). Histologically, those were diagnosed as trabecular hepatoma. Advanced cholangiofibrosis was also noticed. A third of greysh-white round tumor 1 cm in diameter, was found occupying the 3rd and 4th lobes. This was also diagnosed as adenohepatoma. Metestases were observed in the spleen and in the right lung.

Case 5: This rat was sacrificed on the 420th day. Tumor of 1.5 cm in diameter with depressed surface was found in the 6th lobe of the liver, which was histologically a typical trabecular hepatoma developed over adenomotous hyperplasia. Slight cholangiofibrosis and cystic dilatation of the bile ducts were also observed.

Case 6: Sacrificed on the 420th day. In the area painted with 4-NQO solution, flat fibrosarcoma of 1.5 cm in diameter was observed. A white liver tumor of 0.5 cm in diameter was found in the 2nd lobe. Histological examination proved this tumor as an early stage of trabecular hepatoma.

*Group 4.* (Six months' 4-NQO painting followed by 3 months' DAB feeding): Fourteen rats died between 160th and 347th day, among which 4 died from fibrosarcoma which had developed in the painted area. The others died due to pneumonia. The last one was sacrificed on the 420th day. Histological examination of the liver revealed conspicuous central necrosis in 6 cases. Marked cystic dilatation of bile ducts and periportal fibrosis were observed in 5 and 7 cases respectively. There was no development of liver cancer in this group.

*Group 5.* (Six months' 4-NQO painting): Eleven rats died between the 171st and 345th day among which 5 died with fibrosarcoma at the painted area. No liver cancer was found. The most remarkable finding of the liver in this group was the presence of marked central liver cell necrosis. In one case which died on the 269th day, multiple foci of necrosis of the liver cells were observed. Moderate, reparative, bile duct proliferation was found in some cases which died on later experimental days.

**Fibrosarcoma production at the site of 4-NQO painting.** Rats are known to be highly resistant to skin carcinogenesis by painting with carcinogenic hydrocarbon. It was, therefore, with some surprise that a high incidence of skin fibrosarcoma

was noted in the above experiment at the site of 4-NQO application, regardless of whether DAB was fed or not. The following summary may be given concerning the fibrosarcoma production.

In group 3 the first skin tumor appeared on the 210th day 4 months after the initial painting. This rat (Figs. 5 and 6) died on the 360th day due to multiple metastases of the fibrosarcoma to various organs, i.e., spleen, right renal cortex, liver, lung and axillar lymph-nodes. There were 21 rats alive on the 210th day, including the one just described, among which 6 were found bearing fibrosarcoma at the sites of painting area. Among the six, 2 were associated with liver cancer.

In group 4, 6 months' DAB feeding. The skin tumor was first observed on the 260th day. This rat died on the 347th day, was free from liver cancer. Among the remaining rats surviving on the 260th day, animals revealed fibrosarcomas in the painted area. One of those 5 was the only survivor on the 420th day. There was a rat which showed two independent tumor nodules developing in the same painted area; one $3\times2.5$ cm mass in the periphery of, and another $1.0\times1.0$ cm tumor in the central part of a healed ulcer, both of which histologically characterized as fibrosarcoma.

In group 5, treated with 4-NQO painting only, the first tumor was noticed in the painted area on the 195th day. Among those surviving up to the 195th day, fibresarcoma was recorded in 4 rats. Metastasis to regional lymph-nodes was observed in two cases.

Table 1. Tabulation of the total incidences of cholangiofibrosis, adenomatous hyperplasia and adenoma, and liver cancer in the five experimental groups.

| Groups | Treatments | | | Number of rats surviving over 160 days | Cholangiofibrosis | Adenomatous hyperplasia and adenoma | Liver carcinoma |
|---|---|---|---|---|---|---|---|
| | DAB feeding (months) | MC or 4-NQO painting (months) | Total amt. of DAB, mg. (Min.-Max.) | | | | |
| 1. DAB | 3 | 0 | 520.8 (514–521) | 23 | 2 | 0 | 0 |
| 2. DAB +MC | 3 | 6 | 509.6 (500–522) | 19 | 3 | 3 | 1 |
| 3. DAB +4-NQO | 3 | 6 | 517.5 (510–522) | 26 | 9 | 6 | 6 |
| | 4-NQO painting | DAB feeding | | | | | |
| 4. 4-NQO +DAB | 6 | 3 | 510.4 (502–516) | 15 | 1 | 0 | 0 |
| 5. 4-NQO | 6 | 0 | 0 | 11 | 0 | 0 | 0 |

## Discussion

In the present study, it was very remarkable that 6 animals (23%) of Group 3, and one of Group 4, to which 4-NQO and MC, respectively, were applied cutaneously after DAB feeding in total dose of 500 mg per rat for 3 months, developed hepatic carcinoma, while no liver carcinoma was revealed in any of the other groups which were treated with 4-NQO or MC painting alone or in combination with DAB feeding in the reverse order. Group 5, which received 4-NQO painting only, merely showed some degenerative changes of the liver cells without remarkable proliferative changes. DAB feeding alone at the same dosage level was short of inducing hepatic cancer, and none of the 23 rats in this group (Group 1) developed cancer, in agreement with the results previously reported by Odashima[9]. Thus, the present data indicate that the effect of submanifestational dose of DAB can be brought to manifestation (liver cancer production) by additional cutaneous applications of 4-NQO or MC. In this action 4-NQO appears to be definitely more potent than MC; 6 of 26 rats treated with skin painting of 4-NQO developed cancer of the liver while only one of 19 did so with MC. The mechanism of this remarkable remote effect of 4-NQO skin painting on the incidence of hepatic carcinoma in the rat previously fed with inadequate amount of DAB is yet to be analyzed.

It is possible that when 4-NQO is applied to the skin, a small fraction of it is absorbed and carried to the liver. The amount of 4-NQO reaching the liver must be inferred to be very small because of the ready reactivity of the carcinogen with sulphydryl compounds which may be expected to convert some of it into inactive thio-derivative en route. The alternative possibility is that such S-substituted derivatives may be weakly carcinogenic, though so weakly that they fail to produce tumors under the usual conditions of carcinogenesis experiments (Nakahara and Fukuoka). From either of these points of view the experimental results may permit the interpretation that the remote effect on the liver of percutaneously applied 4-NQO summates with hepatocarcinogenic effect of the aminoazo dye.

Yet another possibility may be suggested in view of the findings of Menkin[5], who has been studying the possible role of the inflammatory exudate in carcinogenesis and succeeded in demonstrating the active factor, which is reported to be a nucleopeptide. Since many cells of the skin are injured in loco by 4-NQO painting, it is conceivable that inflammatory exudate liberated from the injured skin tissue may reach the liver by blood circulation and show some "co-carcinogenic" effect on the liver cells. It must be pointed out, however, that in order to validate this interpretation it is first of all necessary to prove that inflammatory exudate itself has carcinogenic action, however small it may be.

## Summary

Using the p-dimethylaminoazobenzene feeding at a low level (about 500 mg per

rat) where there is no liver cancer production, cutaneous applications of 20-methylcholanthrene, but especially of 4-nitroquinoline N-oxide, were shown to bring about the development of liver cancer. Skin painting with 4-nitroquinoline N-oxide or of 20-methylcholanthrene alone failed to produce liver cancer. These results were discussed from the point of view of the summation theory of carcinogenesis, and it was suggested that a small and yet adequate amount of 4-nitrequinoline N-oxide, or its weakly carcinogenic metabolite may reach the liver and complete the submanifestational carcinogenic process already started by p-dimethylaminoazobenzene feeding. The possible "co-carcinogenic" role of inflammatory exudate from the injured skin tissue is not entirely excluded.

It was also demonstrated that 4-nitroquinoline N-oxide is capable of producing skin fibrosarcoma in a high proportion of rats at the site of painting, an unexpected finding in view of the generally accepted fact that the rat skin is insusceptible to tumor producing action of other powerful carcinogens.

The author wishes to express his thanks to Dr. Kunio Oota, under whose guidance the present work was done. Thanks are also due to Dr. Waro Nakahara for his interest and encouragement throughout this study.

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## Explanation of Figures (Plates XI and XII)

All the figures refer to Group 3, in which rats were fed with DAB diet for 3 months, then received 4-NQO skin painting for the following 6 months on basal diet.

Fig. 1. Liver of rat (case 1), died on 160th day, showing the second and 3rd lobes almost entirely occupied by tumor nodules.

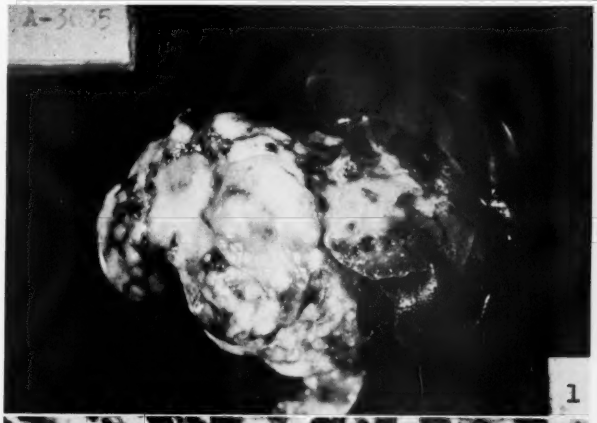
Fig. 2. Microscopic picture of a tumor in Fig. 1, an example of hepatoma with tubulo-glandular pattern. H. & E. ×200

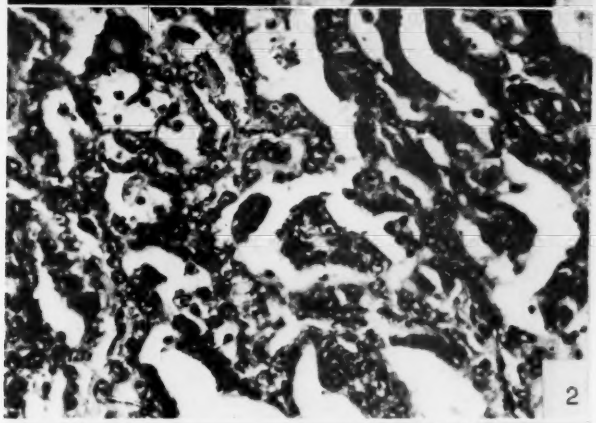
Fig. 3. Liver of rat (case 4) which died on 398th day.

Fig. 4. Histological picture of a tumor in Fig. 3, showing trabecular hepatoma. H. & E. ×200

Fig. 5. Rat (case 3) on 360th day, showing cutaneous tumor at the site of 4-NQO painting.

Fig. 6. Histological picture of the tumor in Fig. 5, showing typical fibrosarcoma. H. & E. ×200

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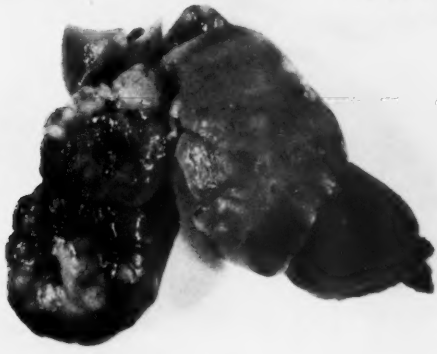

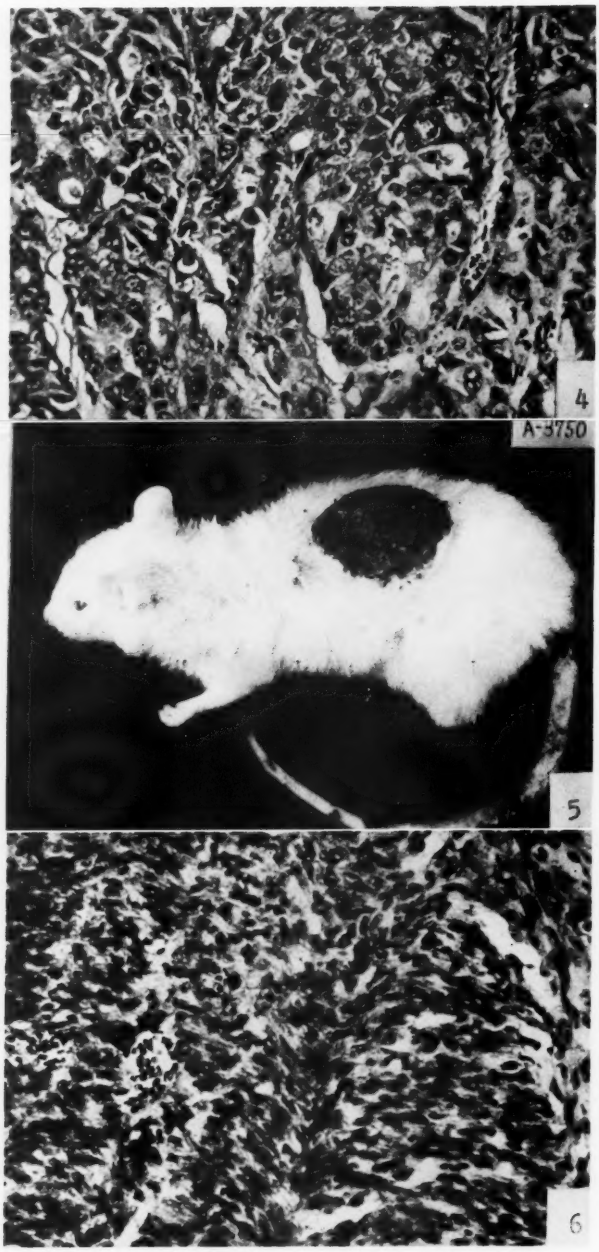
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[GANN, 52, 173~177 ; June, 1961]

# HISTOCHEMICAL STUDIES ON NUCLEAR INCLUSION IN TISSUE CULTURE CELLS INDUCED BY 4-NITROQUINOLINE N-OXIDE

**(With Plates XIII and XIV)**

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## INTRODUCTION

In our previous paper[1], it was reported that 4-nitroquinoline N-oxide (4-NQO) produced characteristic intranuclear inclusion bodies surrounded by halo in tissue culture cells, and that the ability of the 4-NQO derivatives to produce the inclusion bodies was coincident with their carcinogenic action, suggesting some close relation of this inclusion formation to carcinogenesis with this compound.

From the histochemical point of view, it was strongly suggestive that this inclusion might be composed of ribonucleic acid, since it was not stained with Feulgen nor PAS but with eosin and pyronine. However, it was concluded tentatively that the inclusion might consist of basic protein or depolymerized DNA as its pyronin stainability still remained after the treatment with RNase.

It is a well known fact that section of tissue material, in general, is less stainable after the treatment with RNase. In fact, in our repeated experiments, the pyronine stained specimens previously digested with RNase were not sufficiently well stained to bring forth a definite conclusion about the chemical nature of the inclusion. However, when cold perchloric acid[3),4)] instead of RNase was used for digesting RNA, much clearer results were obtained. Furthermore, we have observed that by changing the methods of fixation the intranuclear inclusion took different morphological figures from those previously reported. From these facts an attempt will be made in this paper to elucidate the true constitution of the intranuclear inclusion body surrounded by halo, as originally observed by us.

## MATERIAL AND METHODS

The tissue culture cells used in this experiment were human liver cells (Chang).

The experimental methods for the formation of nuclear inclusion bodies were described in the previous report[1]. The concentration of the inoculum was approximately $10^5$ cells per ml. After 4 or 5 days of incubation the old medium was removed and 0.9 ml of fresh medium was poured in to replace it and 0.1 ml of $10^{-4}$M

4-NQO solution (alcohol concentration 1%) was pipetted into a square culture tube. After 24 hours incubation, the cover slip on which the cultured cells formed monolayer was taken out from the culture tube and was gently washed twice with physiological saline, fixed with ethanol-ether (1 : 1) or with dryice-acetone. In the latter case, the material after the fixation with dryice-acetone was immediately lyophilized and subsequently embedded in paraffin. The fixed materials were stained with hematoxylin-eosin or with methylgreen-pyronine.

In order to perform the fixation and the staining at the same time, acetogentian-violet staining[2] was used. The cover slip was taken out from the culture tube and gently washed with physiological saline. Subsequently, the cover slip preparation was stained with acetogentian-violet solution and sealed with balsam paraffin. Both ether-alcohol fixed and lyophilized materials were treated with 5% cold PCA at 4°C for 24 hours. These materials were also stained with hematoxylin-eosin or with methylgreen-pyronine.

## Results

As reported previously, 4-NQO has a definite ability of producing intranuclear inclusion bodies surrounded by halo in monolayer-cultured Chang's liver cells, when applied in the final concentration of $10^{-5}$M, for 24 hours, at 37°C (Fig. 1). Needless to state that no remarkable change was observed in the cells of control tubes, as shown in Fig. 2. However, this typical picture of intranuclear inclusion bodies surrounded by halo was observed only when the specimen was fixed with ether-alcohol, while one of somewhat different character was seen, as shown in Fig. 3, when the cultured cells were fixed by freezing with dryice-acetone. Namely, with this latter method of fixation, no eosinophilic dot surrounded by halo was seen any more but only several vacuoles filled with homogeneous, eosinophilic mass were demonstrated.

Fig. 4 shows the picture from the specimen fixed by freezing and stained with methylgreen-pyronine, where the contents of the vacuoles were stained deeply with pyronine.

Fig. 5 is a microphotograph taken from the ether-alcohol fixed specimen which was stained with hematoxylin-eosin after the treatment with cold PCA. The eosinophilic dot of the typical inclusion bodies is no more seen to remain.

Fig. 6 shows the disappearance of the pyronine-stained vacuolar contents after the treatment with cold PCA.

Fig. 7 is the picture from the specimen which was fixed and stained with acetogentian-violet after being washed with saline solution. Several vacuoles are noticeable in the cell nuclei showing very similar picture to the one which was fixed with dryice-acetone. The typical figure of the inclusion body consisting of the dense dot

surrounded by halo, which is usually observed in ether-alcohol fixed specimen, is not demonstrable with this method.

Fig. 8 is presented here as the control for Fig. 7, the picture of untreated Chang's liver cells fixed and stained with acetogentian-violet.

## Discussion

From the facts that the intranuclear inclusion bodies produced in tissue culture cells by 4-NQO were eosinophilic, PAS negative, Feulgen negative, and pyronine positive, the RNP nature of the inclusion was strongly suggestive. However, the pyronine stainability seemed to still remain after the treatment with RNase, and it was for this reason that we have in the previous paper tentatively concluded that the inclusion may not consist of RNA but of depolymerized DNA or basic protein, though without any positive proof. As already stated, the stainability of the section of tissue material is generally impaired by RNase pretreatment and this fact was also confirmed from our repeated experiments. We must, therefore, conclude that the judgement about the nature of inclusion by the effect of RNase is not to be accepted without reservation. On the other hand, from the clear cut data obtained by the use of cold PCA described in this report, it is no longer doubtful that the inclusion may consist of RNP, since the cold PCA in our experimental condition does not affect DNA but only RNA. Therefore, our tentative conclusion on the nature of inclusion reported in the previous paper has to be here withdrawn.

That the contents of inclusion may be composed of RNP is also suggested from the results of acetogentian-violet staining. Acetogentian-violet is especially available to clearly demonstrate mitotic apparatus, but nucleolus is, in general, not stained with this solution. This fact and the experimental results that the eosinophilic dense dot situated in the center of the halo, which was formed in the case of ether-alcohol fixation and hematoxylin-eosin staining, was not observed in the case of acetogentian-violet, suggest that the inclusion may be made from the same component as the nucleolus on the substantial level.

One of the important problems before us may be to establish the kind of relationship which exists between the inclusion and nucleolus. In the previous paper, it was described that the inclusion was formed independently of nucleolus, but the subsequent experiments showed such findings as to imply some correlation between the two, or to suggest the compensatory formation of the inclusion for the decrease or the diminution of the nucleolus. The problem of the exact relation between inclusion and nucleolus is the one to be solved in the future.

Electronmicroscopically the tissue culture cell treated with 4-NQO was impressed with selective damage on nucleus, especially nucleolus, while cytoplasmic organella

was almost unchanged. These findings will be fully described in another paper soon to be published[5].

The effect of 4-NQO on the nuclear metabolism of the cell, particularly on RNA metabolism in the cell nuclei, and correlation between this effect and the mechanism of inclusion formation seem to be an important biochemical process in the analysis of carcinogenesis with this compound.

Another point of interest in the present study is the fact that the intranuclear inclusion produced by 4-NQO takes different forms according to the different fixations; namely, by the fixation with ether-alchohol the typical eosinophilic inclusion surrounded with halo indicated in Fig. 1 was obtained, but by the milder fixation such as the lyophilization, the vacuoles filled with homogeneous eosinophilic contents were seen. Morever, in the case of fixation with osmium and formalin, the inclusion, although they are more difficult to observe microscopically, takes the latter type of figure, while it takes the former type of morphology by Bouin fixation. These results suggest that the inclusion surrounded by halo was formed by the condensation of the contents of the vacuoles, which was homogeneously filled, to its center by the strongly dehydrating fixative, i.e., alcohol.

## Summary

1) When the contents of the intranuclear inclusion produced by a suitable concentration of 4-NQO in tissue culture cells were completely digested with cold PCA, instead of with RNase, it was not stained any more with pyronine and eosin. From this finding the RNP nature of the inclusion was concluded.

2) By the dryice-acetone fixation and subsequent immediate lyophilization, the inclusion was observed as vacuoles which were homogeneously filled with eosinophilic contents. From this result, the morphological constitution of the inclusion surrounded with halo by the ether-alcohol fixation was reconsidered.

3) The fixation and staining with acetogentian-violet were performed with demonstrable results for the specimen treated with 4-NQO. From this finding the nature of the inclusion in relation to the nucleolus was also discussed.

Acknowledgements

We wish to express our hearty thanks to Dr. Waro Nakahara for his interest and encouragement throughout this study.

Our thanks are also due to Dr. Kunio Oota for his kind suggestion.

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## Explanation of Figures (Plates XIII and XIV)

All the figures show Chang's strain of human liver cells in tissue culture.

Fig. 1. Treated with 4-NQO, showing typical intranuclear inclusion bodies each surrounded by halo. Fixed with ether-alcohol and stained with H. & E. (1,450 X)

Fig. 2. Untreated control. Fixed with ether-alcohol and stained with H. & E. Compare with Fig. 1. (1,250 X)

Fig. 3. Treated with 4-NQO, showing intranuclear vacuoles filled with homogeneous eosinophillic material. Fixed with dryice-acetone and lyophilization, and stained with H. & E. (1,450 X)

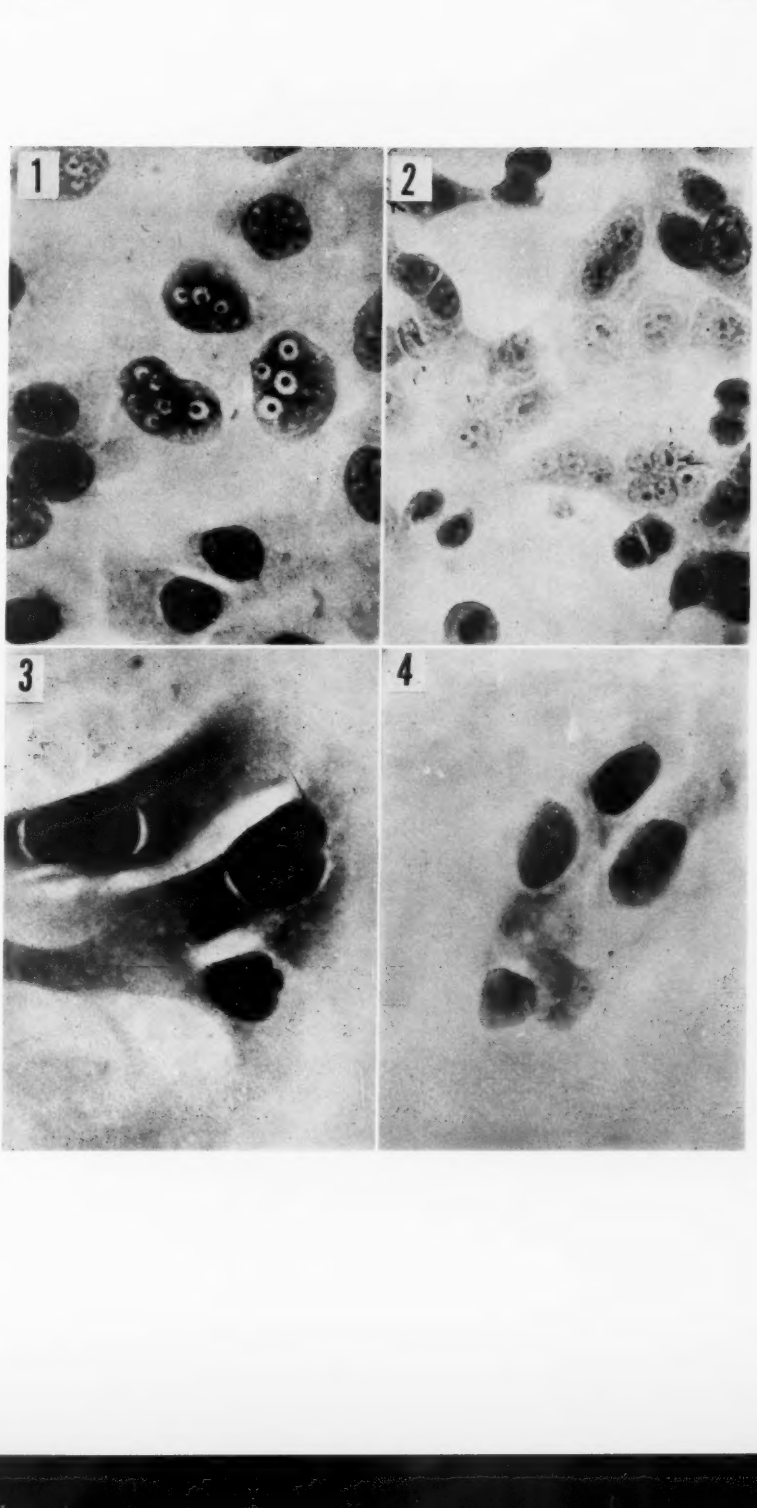
Fig. 4. Treated with 4-NQO, showing positive pyronine staining of the contents of intranuclear vacuoles. Fixed with dryice-acetone and lyophilization and stained with methylgreen-pyronine. (1,450 X)

Fig. 5. Treated with 4-NQO, showing the disappearance of the typical inclusion bodies illustrated in Fig. 1 by the treatment with cold PCA. H. & E. staining. (2,080 X)

Fig. 6. Treated with 4-NQO, showing negative pyronine staining of the vacuolar contents. Fixed with dryice-acetone and lyophilization, treated with cold PCA, and stained with methylgreen-pyronine. (2,080 X)

Fig. 7. Treated with 4-NQO. Fixed with acetogentian-violet solution. (2,520 X)

Fig. 8. Untreated control. Fixed and stained with acetogentian-violet solution. Compare with Fig. 7. (2,520 X)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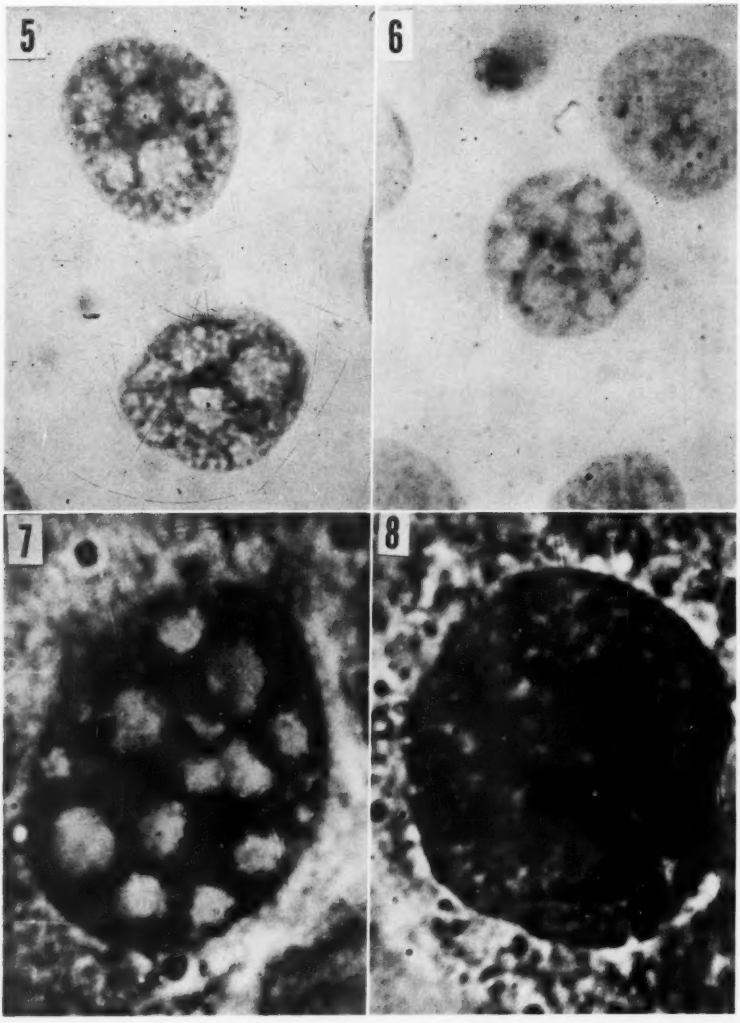
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[GANN, 52, 179~183: June, 1961]

# ISOLATION OF TOXOHORMONE FROM FRIEND VIRUS INFECTED MOUSE SPLEEN

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It is well known that the liver catalase activity of tumor-bearing animals is depressed below that of normal, and this phenomenon has been confirmed by many investigators. In 1948, Nakahara and Fukuoka isolated the so-called toxohormone, which played the role of the depressing factor, from various tumor tissues as active concentrates (1) (2). Some of the chemical characters of toxohormone were reported in our previous papers, and though the finest purification has not yet been attained, our various observations supported the conclusion that it is composed of a polypeptide (2) (3) (4) (5).

Whether or not toxohormone occurs in normal tissues is a question of importance, and some investigators reported that a small amount of it could be isolated from rat or human materials (6) (7) (2). In this paper data on the yield of toxohormone fractions from normal mouse spleen and from the huge spleen of mouse inoculated with the Friend virus are presented. The Friend virus produces a leukemia-like disease in mice, in which an extraordinary enlargement of spleen forms the major pathology during the early period after the inoculation of the virus, followed by a generalized reticulosis and leukemic blood picture (8). The present investigation may be of interest not only as the first study on toxohormone in normal and pathological spleens of the mouse, but also as a contribution to chemical pathology of the Friend disease.

## Materials and Methods

The original Friend virus used in this experiment was the lyophilized specimen kindly presented by Dr. Charlotte Friend, Sloan-Ketterling Institute, New York, and the strain has been maintained in our laboratory through serial passages in female Swiss mice by intraperitoneal injections of 0.1 ml of 10 percent homogenate in physiological saline of the enlarged spleen, according to the direction given by Dr. Friend. In the present experiment, typical, huge spleens were collected from mice three weeks after the inoculation. Normal spleens were collected from 400 untreated mice to serve as control. Both groups of the spleens were used on the same wet weight basis for the preparation of toxohormone fractions.

Crude toxohormone fractions were obtained by the alcohol precipitation of the

heat-stable component of the water extract of tissue homogenates. The partial purification was carried out by the adsorption procedure using calcium phosphate gel at pH 7.0. This preparation, called Ta-fraction, was more active than the original crude toxohormone (5).

The bioassay of the toxohormone activity was carried out by the routine method in this laboratory as described in our previous report (5), measuring the liver catalase activity by von Euler-Josephson's method with some slight modification (9).

In order to provide an accurate basis for comparison of the yields of toxohormone from the two sources, we decided to check them against the desoxyribonucleic acid content (DNA) of the starting tissue materials. For this purpose one gram in wet weight of the tissue was fractionated by the modified Schmidt-Tannhauser-Schneider method (10), and an aliquot of the nucleic acid fraction was used to determine the DNA content by the diphenylamine reaction, from which the total DNA content per gram tissue was calculated.

## Results

**Liver Catalase Activity in Friend Virus Infected Mice.** The organ weights (wet) in Friend virus infected mice at three weeks after inoculation are compared with those of normal mice in Table 1, which shows a slight hepatomegary and greatly enlarged spleen, the organ weights amounting to 1.5 and 16 times those of normal, respectively. At this experimental period the determination of liver catalase activity

Table 1. Weights of liver and spleen from normal and Friend virus infected mice.

| Organs | Normal mice (g) | Friend virus infected mice (g) |
|---|---|---|
| Liver | 0.788±0.074 | 1.201±0.607 |
| Spleen | 0.082±0.019 | 1.290±0.885 |

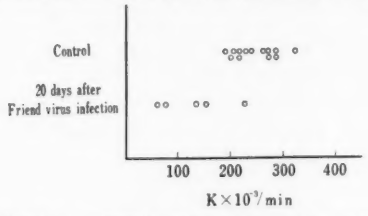


Chart 1. Hepatic catalase levels in Friend virus infected mice.

demonstrated a distinctly decreased level in the virus-infected mice, as shown in Chart 1. The decreased enzyme level cannot be considered as due to the increased liver weight as the concentration of liver catalase does not generally change by the slight alteration of the body or organ weight. Histological changes in the liver are of the slightest at this stage of the Friend disease. On the contrary, the result is suggestive of the toxohormone effect originating from the virus-affected spleen.

**Toxohormone Activity of Isolated Fractions.** The crude and partially purified toxohormone fractions isolated from various tissues were assayed by the depression of liver catalase activity *in vivo*, which was caused when these preparations were

injected intraperitoneally into normal mice.

As shown in Chart 2, crude toxohormone fraction from normal rat liver was only slightly active, much less so than the rhodamine sarcoma fraction at 75 mg doses. When the fraction from the sarcoma tissue was further purified by adsorption on calcium phosphate gel (Ta-fraction) the preparation was active in 20 mg doses, as we previously reported (11).

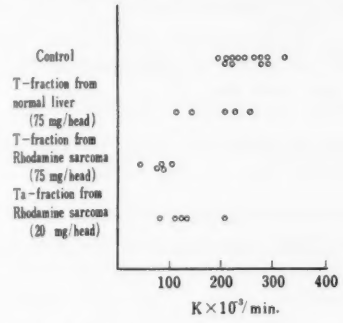


Chart 2. Effects of several toxohormone fractions on the hepatic casalase activities.

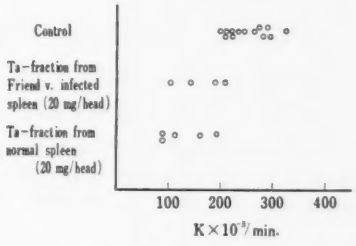


Chart 3. Effects of several toxohormone fractions on the hepatic catalase activities.

The Ta-fractions of normal and Friend disease spleen were assayed at 20 mg doses. The both preparations markedly decreased the catalase activity, and no difference was detected between them. See Chart 2.

**Difference in the Yields of Toxohormone Fraction.** The yields of toxohormone fractions from normal mouse liver and spleen, Friend disease spleen, and rhodamine sarcoma are compared in Table 2. The yields of the crude fraction from normal tissues were 0.5 percent of the starting material, both liver and spleen, and from rhodamine sarcoma and from Friend spleen were 1.5 and 1.7 percent, respectively. The Ta-fraction isolated amounted to about 30 percent of all the crude toxohormone fractions alike.

Table 2. Toxohormone yields from the same unit weight of various raw materials.

| Sources | T-fraction | Ta-fraction* |
|---|---|---|
| Normal liver | 0.5% | 0.17% |
| Normal spleen | 0.5 | 0.17 |
| Friend virus infected spleen | 1.7 | 0.48 |
| Rhodamine fibrosarcoma | 1.5 | 0.42 |

* These fractions were prepared by calcium phosphate gel adsorption method from the respective T fraction (crude).

These quantitative relations of the yields of toxohormone fractions were reexamined by calculating the yield ratios to the total amounts of DNA contained in each tissue. As summarized in Table 3, the total DNA contents per gram tissue weight were almost the same for both normal and Friend spleens, thus

Table 3. Comparison of the yields of crude toxohormone fractions from various tissues as calculated against per unit DNA content.

| Sources | Yields mg per 30 g tissue | Yields mg/DNA μg/ Tissue g | Yields mg/DNA mg |
|---|---|---|---|
| Normal liver (Mouse) | 150 | 5/280/1 | 17.5* |
| Normal spleen (Mouse) | 150 | 5/1,100/1 | 4.5 |
| Friend spleen (Mouse) | 500 | 16.5/960/1 | 17.0 |
| Fibro-sarcoma (Rat) | 450 | 15/430/1 | 35.0 |

* The large yield from the normal liver does not mean correspondingly great toxohormone content since the liver fraction is of very weak activity.

disposing of any doubt which may arise from the possible difference between them in their cellular and fibrous compositions.

The yields of toxohormone from the enlarged Friend spleen per DNA unit were about four times as much as from the normal spleen, as shown in the last colum in Table 3.

## Discussion

Although there is no doubt that fractions with toxohormone activity can be extracted from variety of normal tissues, the active substance has been regarded as of no special significance beyond possiblly maintaining the normal liver catalase level where it is. This means that the cause of the depression of liver catalase activity in tumor-bearing animals should be looked for in the toxohormone of tumor tissue, and the reported occurrence of toxohormone in urine and blood of cancer patients shows that the substance is thrown into the circulation from the tumor. No indication exists that blood or urine of normal individuals contains a similar active substance.

It is logically possible that the toxohormone of tumor tissue may be one and the same substance as the normal tissue "toxohormone", only produced in greatly increased amounts, or that it represents two different chemical entities which become concentrated in the same fraction. This important question can be ultimately settled by adequate purification of toxohormone from normal and from tumor tissues, which should yield either a single substance or two separable ones.

Whichever the point of view one may adopt, the fact that normal spleen and the Friend disease spleen both yield toxohormone fractions of approximately equal activity but in greatly different amounts, as clearly demonstrated in the present study, suggests that the splenomegary of the Friend disease approaches malignancy in the sense that the protein metabolism in the enlarged spleen tends towards that of tumor tissue, at least insofar as the greatly increased production of a characteristic protein-like substance indicates.

## Summary

Toxohormone was separated from normal mouse spleen and from the enlarged spleen of the Friend virus infected mice, three weeks after the inoculation of the virus. Two corresponding fractions from the two sources revealed about the same activity gram for gram, but the Friend spleen yielded each fraction in amount about four times as much as from normal spleen per unit fresh material. The yield ratio was checked against the DNA content of the tissue materials from which the fractions were separated.

These findings demonstrate a greatly increased production of toxohormone by the Friend virus infected spleen and suggest that the huge spleen in this disease may be regarded as approaching malignancy in its peculiar protein metabolism.

The author's sincere thanks are due to Dr. Waro Nakahara for his stimulating encouragement, and to Drs. Fumiko Fukuoka, Takashi Sugimura, Tsutomu Kasuga, Yasuhiko Shirasu, and Motoo Hozumi for their helpful discussion. He also wishes to thank Mr. Kiyoshi Noguchi for his technical assistance.

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[GANN, 52, 185~189; June, 1961]

# GLYCOLYSIS OF SPLEEN OF MICE INFECTED WITH FRIEND VIRUS

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The Friend virus is known to cause the pronounced reticulum cell proliferation and erythroblastosis in the spleen of the mice of Swiss, DBA and RF strains[1-4]. In view of the rapid enlargement of spleen and similarity of this disease to the malignant leukemia, it seems to be interesting to compare the glycolytic activity of the Friend virus infected spleen with that of the normal one of the mice. In this paper, the data on aerobic and anaerobic glycolysis and on the respiratory activity are described.

## EXPERIMENTAL

Female mice of the Swiss strain were used throughout. They were five to six-week old at the time of virus inoculation. Mice were maintained on whole grains of wheat supplemented with dried fish and dried alfalfa.

Friend virus was sent to Dr. Waro Nakahara, Director of Cancer Institute, Tokyo, through the courtesy of Dr. Charlotte Friend, Sloan-Kettering Institute, New York. The virus has been maintained in serial passages in Swiss mice.

Enlarged spleens were aseptically removed from the mice on the twenty-first day after the virus inoculation, and the homogenate was made with four volumes of sterilized saline by using Potter-Elvehjem type glass homogenizer. After centrifuging at 3,000 r.p.m. for 10 minutes to remove the cell debris and fibrous material, 0.1 ml of the supernatant was injected intraperitoneally in each mouse. The mice were divided into several groups and animals in each group were sacrificed on a certain day.

Blood was removed by decapitation and the spleen was immediately weighed and chilled. Respiration experiments were done in the conventional Warburg respirometer. About fifty milligrams of slices in wet weight was put into the flasks containing 3 ml of Krebs-Ringer-phosphate buffer, pH 7.4 with 0.2 ml of 10 percent KOH in the center well. No substrate was added. In glycolysis experiments about forty milligrams of slices in wet weight was put into two flasks, containing 3 ml of Krebs-Ringer-phosphate buffer, pH 7.4 with glucose at 0.2 percent. Gas phase in one flask was air and that in the other flask was nitrogen. After incubation at 37°C

for one hour, the reaction was stopped by the addition of trichloroacetic acid to give the final concentration of 5 percent, and an aliquot of the deproteinized supernatant was assayed for lactic acid according to the method of Baker and Summerson[5]. The initial lactic acid level was first determined and this value was subtracted from the one after the incubation. A special care was taken to pick up several slices from the various parts of one spleen to get the balanced sampling. In order to compare the results of the present experimental data with those in other experiments, the lactic acid formation was expressed in terms of microliters of gas per milligram dry weight per hour ($Q^{O_2}_{CO_2}$ and $Q^{N_2}_{CO_2}$) as used generally in the case of manometric method.

As examples of malignant tissue, NF sarcoma of albino mice and Rhodamine sarcoma of albino rats which are fibrosarcomas and easily transplantable, were used for comparison of respiratory and glycolytic activities.

To obtain the enzyme extract for the determination of lactic dehydrogenase and aldolase, the spleen was homogenized with nineteen volumes of physiological saline, and the homogenate was subjected to the sonication for 4 minutes in a sonic oscillator (Tōyōrikō Seisakusho Co., Ltd., Model 50-5), operating at 9 kc, and it was centrifuged at 1,600 g for 20 minutes. Supernatant was properly diluted for enzyme assays. Lactic dehydrogenase was measured by the oxidation of DPNH in the presence of pyruvate[7], and aldolase was assayed by the method of Dounce *et al*[8]. Protein was determined by the method of Lowry *et al*[9].

## Results

The weight increase of spleen after virus inoculation is illustrated in Fig. 1. The weight of spleen on the seventh day was already four to five times as much as that of normal spleen. The spleen continued to enlarge during the three weeks after virus inoculation and remained nearly constant thereafter, provided that there was no splenic rupture in the mean time. The dry weight percentage of these spleens is almost the same as that of normal ones, as shown in the second column in Table 1.

The respiratory activity of the virus infected spleen did not differ from that of normal spleen as shown in the third column of Table 1. The sarcomas gave lower respiratory activity than the spleen.

A slight increase in the aerobic and the anaerobic glycolysis was observed in the

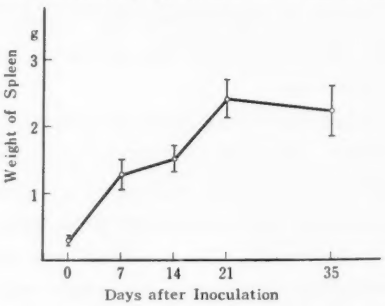


Fig. 1. Increase in Weight of Spleen after Friend Virus Inoculation.

Table 1. Respiratory and Glycolytic Activity of Normal and Friend Virus Infected Spleen.

| | Dry Weight Percent | Respiratory* Activity | Glycolytic Activity** | |
|---|---|---|---|---|
| | | | Aerobic | Anaerobic |
| Days after inoculation | | | | |
| 0 (Normal) | 21.7±0.2 | 4.92±0.95 | 3.75±0.52 | 7.15±0.92 |
| 7 | 20.6±0.1 | 3.87±0.62 | 4.29±0.20 | 6.33±0.79 |
| 14 | 20.6±0.2 | 4.59±0.66 | 4.39±0.36 | 7.40±0.40 |
| 21 | 21.0±0.3 | 4.20±0.57 | 4.58±1.17 | 11.16±1.68 |
| 35 | 20.7±0.4 | 4.87±0.64 | 5.17±0.45 | 9.30±0.44 |
| Rhodamine Sarcoma | 16.3 | 3.42 | 10.84 | 17.88 |
| NF Sarcoma | 16.5 | 2.75 | 16.96 | 23.00 |

* $Q_{O_2}$, $\mu$l oxygen consumption per mg dry weight per hour.

** $Q^{O_2}_{CO_2}$ and $Q^{N_2}_{CO_2}$, $\mu$l acid formation per mg dry weight per hour under air or nitrogen. Each figure represents the average value of five or six spleens and that of three samples of sarcomas.

Table 2. Lactic Dehydrogenase and Aldolase Activity of Normol and Friend Virus Infected Spleen

| | | Lactic Dehydrogenase* | Aldolase** |
|---|---|---|---|
| Normal | 1 | 615 | 466 |
| | 2 | 610 | 377 |
| Friend Virus Infected Spleen | 1 | 625 | 501 |
| | 2 | 590 | 507 |

* Activity is expressed as $1,000 \times \Delta E_{340}$ per min per mg protein at 24°C.

Reaction Mixture: 0.2 ml of 0.01M sodium pyruvate, 0.4 ml of 0.002M DPNH, 2.3 ml of 0.1M $KH_2PO_4$-$K_2HPO_4$ buffer (pH 7.4), 0.1 ml of enzyme extract containing 20 to 30 $\mu$g of protein, Total volume 3.0 ml.

** Activity is expressed as $1,000 \times E_{585}$ per mg protein after colour development with Baker & Summerson's procedure on aliquot of reaction mixture.

Reaction mixture: 0.5 ml of 0.01M sodium fructose diphosphate (pH 7.2), 0.1 ml of 0.2M collidine buffer (pH 7.2), 0.1 ml of 0.056M hydrazine sulfate (pH 7.2), 0.1 ml of 0.002 M sodium iodoacetate, 0.1 ml of enzyme extract, containing 100 to 150 $\mu$g of protein. Incubated at 24°C for 20 min, Added 4.0 ml of 8% trichloroacetic acid. 1.0 ml of deprotenized solution was tested for colour reaction.

virus infected spleen, though the measured value was quite lower than that of the sarcomas.

The results on assay of lactic dehydrogenase and the aldolase in the extract of the normal and the virus infected spleen at the fifth week after the inoculation are given in Table 2. Lactic dehydrogenase activities in the virus infected and the normal spleens are almost the same, and aldolase activity shows a slight increase in the virus infected spleen. These results are coincident with the data of the glycolysis experiments using slices.

## Discussion

Since the original works of Warburg in 1923[10], it is well established that tumor tissues have high rate of lactate production in the aerobic and anaerobic condition. The data collected by Aisenberg[11] from many workers' reports indicate that the anaerobic glycolysis of animal tumors is consistently in excess of 20 when expressed in the value of $Q_{CO_2}^{N_2}$ and that $Q_{CO_2}^{O_2}$ value of the aerobic glycolysis of most tumor tissues is more than 10.

The results of the present experiments show that $Q_{CO_2}^{N_2}$ value of Friend virus infected spleen remains around 10 during the five weeks after the virus inoculation, and $Q_{CO_2}^{O_2}$ value is not in excess of 6. On the contrary, the glycolytic activity of two sarcomas, investigated as examples of maligmant tumors, shows a high rate of the aerobic and the anaerobic glycolysis as typical malignant tumors. It is needless to mention that the transplantable fibrosarcoma tissues consist almost entirely of malignant cells, while, on the other hand, the enlarged spleen of the Friend disease contains various kinds of cells, some of which are certainly normal cells as reported to be erythroblasts and myeloblasts by Furth *et al.*[12] and Kasuga *et al.*[13] This coexistence of the normal cells might be partly responsible for the absence of a marked difference of glycolytic activities between the normal and the infected spleen.

It would be worth while to consider carefully the reports on glycolytic activity of homologous series on normal and neoplastic tissues. Though azo-dye induced rat hepatoma had the increased glycolysis, the studies on spontaneous mouse hepatoma showed that the increased rate of aerobic and the anaerobic glycolysis was found in only one of six such hepatomas[14]. Hall and Furth reported the elevated glycolysis in spontaneous and transmitted lymphoid leukemia of mice[15]. On the other hand, Burk *et al.* described that the anaerobic glycolysis of the spleen and lymphnodes of mice with radiation- and methylcholanthrene-induced leukemia were not elevated over that of the normal spleen[16]. These findings suggest that some of the malignant tissues have higher glycolytic activity than the normal tissues of origin, while others have not such an enhanced glycolytic activity. The growing cells in the enlarged spleen infected with Friend virus might belong to the latter class of malignant tumors, or they might not be malignant in the strict sense at least during the first five weeks after the virus inoculation.

Although the present experiment was limited for five weeks after the virus inoculation, it would be possible that the glycolysis might increase in the advanced stage after this period. Accordingly it might be desirable to investigate the glycolytic activity of transplantable solid tumor variants, as established from grafts of the Friend virus infected spleen by Buffet and Furth[12] and Friend and Haddad[17].

## Summary

The respiratory and glycolytic activities of Friend virus infected spleen were investigated for the first five weeks after the virus inoculation. The respiratory activity of the virus infected spleen was the same as that of the normal one. Aerobic and anaerobic glycolysis showed a slight increase on the third to fifth week after the virus inoculation. These results were briefly discussed.

## Acknowledgements

We are grateful to Dr. Waro Nakahara for his advice and interest in this work and are indebted to Dr. F. Fukuoka for supplying us with NF sarcoma. We express our graditute to Dr. T. Kasuga for his valuable criticism.

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[GANN, 52, 191~196; June, 1961]

# ANTI-TUMOR EFFECT OF SOME NITROGEN MUSTARD PEPTIDES

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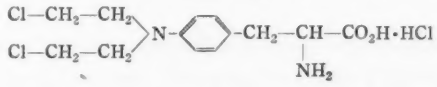
The concept of nitrogen mustard derivatives with latent activity, consisting of a structure that will maintain a reduced reactivity of the alkylating group until the molecule is broken down, has led to the introduction of phenylalanine nitrogen mustard (melphalan (1) and sarcolysine (2)) and later to its peptides as potential cancer chemotherapeutic agents. It has been demonstrated that the inhibiting effects of these compounds on tumors differ very markedly, depending upon the kind of peptides, thus emphasizing the important role of the carrier moiety in anti-tumor action (3, 4). In the present study attempts were made to obtain additional data bearing upon this interesting point.

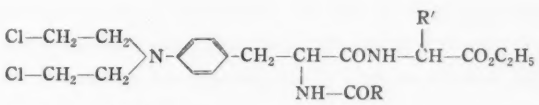
## MATERIAL

All the compounds tested were prepared in the laboratory of the Fujisawa Pharmaceutical Company, Osaka, by Dr. Kazuo Nakanishi. They were:

1. *p-di*-(2-chloroethyl)-amino-pheynylalanine. [S]



2. N-formyl-*p-di*-(2-chlorethyl)-amino-phenylalanyl-phenylalanine ethyl ester. [F-Phe]



R=H, R′=$CH_2C_6H_5$

3. N-formyl-*p-di*-(2-chloroethyl)-amino-phenylalanyl-valine ethyl ester. [F-Val]

R=H, R′=$CH\langle^{CH_3}_{CH_3}$

4. N-acetyl-*p-di*-(2-chloroethyl)-amino-phenylalanyl-valine ethyl ester. [A-Val]

R=$CH_3$, R′=$CH\langle^{CH_3}_{CH_3}$

5. N-formyl-*p-di*-(2-chloroethyl)-amino-phenylalanyl-glycine ethyl ester. [F-Gly]
R=H, R′=H

6. N-acetyl-*p*-*di*-(2-chloroethyl)-amino-phenylalanyl-glycine ethyl ester. [A-Gly]

$R=CH_3$, $R'=H$

7. N-acetyl-*p*-*di*-(2-chloroetyl)-amino-phenylalanyl-leucine ethyl ester. [A-Leu]

$R=CH_3$, $R'=CH_2-CH\begin{matrix}CH_3\\CH_3\end{matrix}$

8. N-acetyl-*p*-*di*-(2-chloroethyl)-amino-phenylalanyl-isoleucine ethyl ester. [A-Isoleu]

$R=CH_3$, $R'=CH\begin{matrix}CH_3\\CH_2-CH_3\end{matrix}$

In all these peptides amino acids were of *dl*-form. Sarcolysine peptides are all insoluble in water, difficultly soluble in petroleum solvents, and soluble in alcohol, acetone, ether, etc.

## Experimental Results

**Effect on the Viability of Tumor Cells in vitro.** Notwithstanding the objection from som groups of investigators, experience has shown us that *in vitro* tests on the cancericidal action could yield some indication of the value of chemicals as possible anti-cancer agents, especially in dealing with compounds which are not anti-metabolites. The *in vitro* tests were carried out according to the method which was designed by Fukuoka (5) and which has been routinely used in this laboratory for some years.

Approximately the same amount of the Nakahara-Fukuoka mouse sarcoma tissue was cut up into fragments of about 1 mm. diameter, and was immersed in suspensions of test substances varying in concentrations from 0.05 percent 0.005 percent and 0.0005 percent in physiological salt solution, using the plain salt solution for control. After standing at about 4°C for 24 hours, tumor fragments from the four lots were inoculated subcutaneously into four different sites of one mouse. Four mice were used in each test and the growth of the tumors was observed for two weeks or more.

The result of the experiment is shown in Table 1, from which it is evident that sarcolysine, both *l*- and *dl*-forms, kills tumor cells at the high dilution of 0.0005 percent, and glycine peptide, acetyl valine peptide and leucine peptide at 0.0005 percent, at which percent formyl valine peptide and isoleucine peptide prevent the growth of more than half of the tumors but not all, while phenylalanine peptide is ineffective even at the lowest dilution of 0.05 percent. Whether the observed difference in the effect among glycine, leucine, isoleucine and formyl and acetyl valine peptides is to be considered significant or not is a moot point. There is no doubt, however, that these as a group, stand intermediate between sarcolysine, which is very highly

effective, and phenylalanine peptide, which is least effective.

**Toxicity by Per Os Administration.** Preliminary toxicity tests by intraperitoneal injections of suspensions showed that there is a striking difference between sarcolysine and its peptides, $LD_{100}$ being somewhere between 25 to 50 mg/kg for the former contrasted to about 4 g/kg for most of the latter. It is practically impossible to deliver anything like the maximum tolerated amount of the peptides, with such a low toxicity, into animals by injection.

$LD_{50}$ as determined on mice by a single administration *per os* of the compounds is as follows:

| | |
|---|---|
| S (dl-form) | 25～30 mg/kg |
| A-Gly | 200～250 mg/kg |
| F-Gly | 750～1000 mg/kg |
| A-Val | 800～1000 mg/kg |
| F-Val | over 4000 mg/kg |
| F-Phe | over 4000 mg/kg |

Table 1. Cancericidal action *in vitro*.

| Compounds | Concentrations of suspension, % | | |
|---|---|---|---|
| | 0.05 | 0.005 | 0.0005 |
| S(l-Sarcolysine) | + | + | + |
| S(dl-Sarcolysine) | + | + | + |
| F-Phe | − | − | − |
| A-Gly | + | + | − |
| F-Val | + | ± | − |
| A-Val | + | + | − |
| A-Leu | + | + | − |
| A-Isoleu | + | ± | − |

In the above table, − indicates that all the tumors grew (no effect); ± that more than half of the tumors failed to grow, but some did; and + that none of the tumors grew (complete killing of tumor cells).

**Effect on the Survival of Mice bearing Ehrlich Ascites Carcinoma.** Freshly aspirated Ehrlich carcinoma ascites (one week after inoculation) was injected intraperitoneally in amounts corresponding to about ten million cells into normal male mice of dd strain, 5 weeks of age and weighing about 20 g at the time of the first treatment. They were treated with administration *per os* of the test compounds, starting at 48 hours after the ascites injection and continuing daily for 5 consecutive days. The dose per day per mouse corresponded to 1/4 to 1/5 of the $LD_{50}$, suspended in 0.1 to 0.4 cc of water for each administration. Control mice received the same amounts of water.

The results are shown in Chart 1, in terms of the length of survival periods. It may be seen that the best prolongation of the survival period was obtained with sarcolysine (S) and with formyl glycine peptide (F-Gly), followed by acetyl glycine peptide (A-Gly) and formyl phenylalanine peptide (F-Phe) and acetyl valine peptide (A-Val). Formyl valine peptide (F-Val) showed perhaps the least effect.

**Effect on the Growth of Subcutaneously Implanted Tumors.** These experiments were conducted under the identical conditions to the preceding ones, except that the tumor (NF sarcoma) was implanted subcutaneously, and that the effect of the treat-

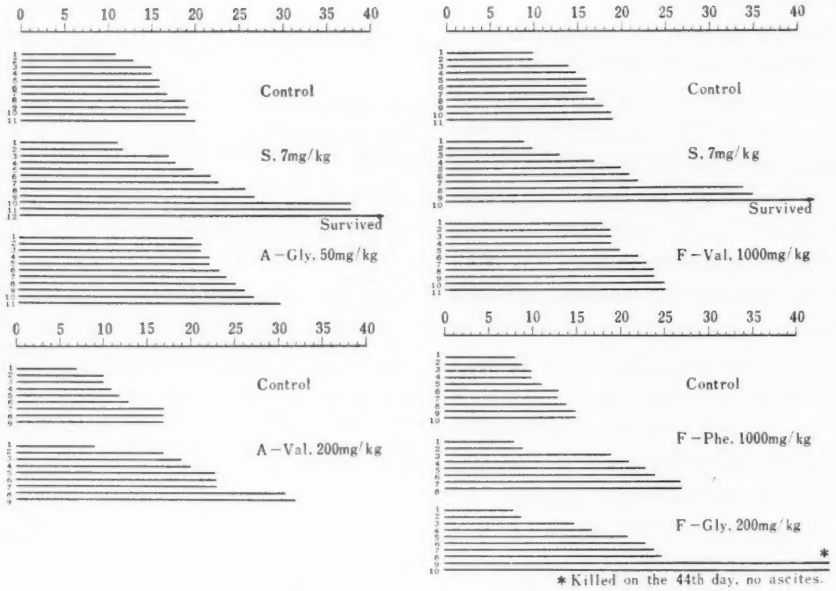



Chart 1. Effect of administration *per os* of several sarcolysyl peptides on the survival period of mice bearing Ehrlich ascites carcinoma, in comparison with that of sarcolysine.

Table 2. Effect of administration *per os* of several sarcolysyl peptides on the growth of subcutaneously implanted tumors. (NF sarcoma and Sarcoma 180).

| Compounds | Dose mg/kg | NF sarcoma | | | | | Sarcoma 180 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Av. body wt. change (g) | | Av. tumor wt. (g) | | Inhibition ratio % | Av. body wt. change (g) | | Av. tumor wt. (g) | | Inhibition ratio % |
| | | Control | Treated | Control | Treated | | Control | Treated | Control | Treated | |
| S | 7 | +1.4 | −4.1 | 1.8 | 0.08 | 95.6 | +0.4 | −3.6 | 4.2 | 0.6 | 85.7 |
| A-Gly | 50 | +1.2 | −1.2 | 1.4 | 0.11 | 92.1 | +0.4 | −0.5 | 4.2 | 1.7 | 59.5 |
| F-Gly | 200 | +3.0 | −3.7 | 1.4 | 0.07 | 95.0 | −0.1 | −5.1 | 3.7 | 0.4 | 89.2 |
| A-Val | 200 (Sar. 180) 250 (NF sar.) | +1.2 | −3.2 | 1.4 | 0.07 | 95.0 | −0.1 | +0.6 | 3.7 | 1.8 | 51.4 |
| F-Val | 250 | +2.5 | −1.7 | 2.2 | 0.2 | 90.9 | +1.0 | −0.8 | 3.6 | 3.2 | 11.1 |
| | 1,000 | +4.8 | −0.2 | 2.1 | 0.4 | 81.0 | −0.1 | −1.1 | 3.7 | 2.3 | 37.8 |
| F-Phe | 1,000 | +3.0 | −4.0 | 1.4 | 0.07 | 95.0 | +0.4 | −4.5 | 4.2 | 0.6 | 85.7 |

ment was judged by the relative sizes and weights of the tumors on the 9th day after implantation, when all the mice were killed and autopsied, each excised tumor being weighed.

A striking inhibition of tumor growth was obtained with all the compounds tested, the average tumor weights in the treated groups being only 1/5 to as small as 1/20 of those in controls. (Table 2).

A parallel series of experimets were carried out using sarcoma 180, with the results shown also in Table 2. With this tumor the effect of the treatment was somewhat less striking and in most cases the average tumor weights in the treated mice amounted to 1/2 to 1/10 of those in control mice. Formyl valine peptide was exceptional in producing scarcely significant effect even at the high dose of 1 g/kg per day.

The relative tumor sizes in two typical experiments are illustrated in Fig. 1: effect of formyl glycine peptide on NF sarcoma and that of formyl phenylalanine pepitde on sarcoma 180.

**Effect of Intraperitoneal Injections of Isoleucine Peptide.** The *in vitro* cancericidal action of isoleucine peptide was noted already (Table 1). Toxicity tests with

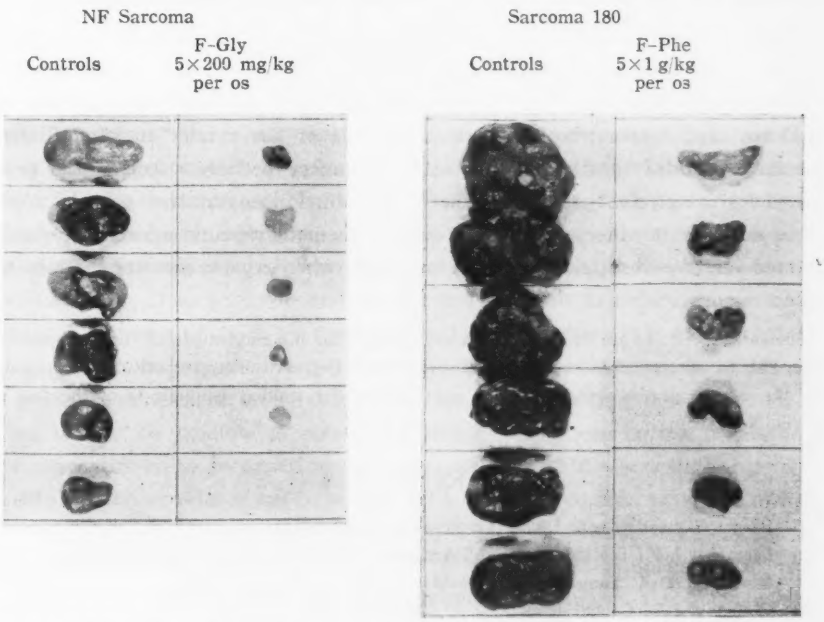



Fig. 1. Relative sizes of tumors in two typical experiments, showing the effect of formyl-sarcolysyl-glycine peptide on NF sarcoma (to the left) and that of formyl-sarcolysyl-phenylalanine peptide on sarcoma 180 (to the right).

mice by intraperitoneal injection established $LD_{50}$ to be 250~300 mg/kg. The substance showed a definite prolongation of the survival period of mice bearing Ehrlich ascites carcinoma, when it was injected intraperitoneally in 50 mg/kg /day dose for 5 consecutive days, beginning the injection 48 hours after the tumor implantation (Chart 2). The growth of the subcutaneous grafts of Nakahara-Fukuoka sarcoma was also inhibited by intraperitoneal injections of this peptide in 50 mg/kg/day dose for 5 consecutive days beginning 48 hours after the tumor implantation. The inhibition ratio was 85.9 percent.

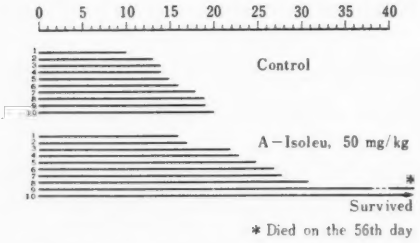



Chart 2. Effect of intraperitoneal injections of acetyl-sarcolysyl-isoleucine peptide on the survival period of mice bearing Ehrlich ascites carcinoma.

These data are not directly to be compared with those of other peptides, because of the different mode of administration. They are quoted here merely to show the hitherto unrecorded anti-tumor effect of the new nitrogen mustard peptide, which, however, was not available to us in sufficient amount to make adequate tests.

## Summary and Comments

The results described above generally confirmed the conclusion arrived at by Larionov, and demonstrated the important role of the carrier moiety of nitrogen mustard peptides in determining their anti-cancer activity. Among the peptides tested formyl glycine peptide, followed by formyl phenylalanine peptide appeared to be superior to others as possible cancer chemotherapeutic agents. It should be pointed out, however, that the results may differ when experiments are conducted with other tumors.

The authors' thanks are due to Dr. Waro Nakahara for his encouragement and support. They also wish to acknowledge their indebtedness to the Fujisawa Pharmaceutical Company, Osaka, for the supply of the samples of the peptides and for making available to us the data of Dr. Kazuo Nakanishi.

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[GANN, 52, 197~202; June, 1961]

# CARCINOSTATIC LIVER FACTOR
## I. EFFECT IN VITRO OF HOMOLOGOUS LIVER EXTRACT ON VIABILITY OF EHRLICH ASCITES CARCINOMA CELLS

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Histopathology of human tumors has long since established that there is at the growing edge of malignant tumors frequent liberation into the surrounding tissues of isolated tumor cells, the phenomenon of the so-called disjunction. In recent years clinical studies have come to widely recognize the occurrence of tumor cells in the peripheral blood of cancer patients, often in no negligible numbers. Considering these facts, one may wonder why the actual metastasis formation does not take place more frequently than it does. It would appear that cancer cells liberated into the circulation are largely killed or otherwise disposed of before they establish themselves in some distant organ sites. The host body, at least during early stages of the disease, may be assumed to possess a mechanism by which these isolated cancer cells are destroyed. The present study has been undertaken upon the basis of this assumption.

It is self-evident at the outset that, even if such a mechanism exists, which disposes of isolated cancer cells in body fluid, the direct experimental demonstration of the fact would be extremely difficult. As a matter of fact it is a common knowledge that under ordinary conditions normal mouse blood is without effect on the viability of mouse tumor cells, showing that the concentration of the hypothetical carcinostatic factor, if sufficient to destroy few isolated cells in nature, is too low to affect such number of cells as must be used in any practicable experimental procedure. It can be supposed, however, that the carcinostatic factor in question may by produced somewhere in the body and thence thrown into the circulation, and, if so, it may be possible to obtain the factor from some normal organs in sufficient concentration to be experimentally assayable. The idea is perhaps not too far-fetched since the growth of rat hepatoma cells in tissue culture is known to be somewhat inhibited when liver extracts are added (1). Also, Herbut and Kraemer's (2) recent demonstration of the inhibition *in vivo* of mouse lymphosarcoma by certain heterologous liver extracts makes it advisable to examine the homologous liver extract-tumor cell interaction.

Our recent experiments showed that the above assumption may be correct. It was discovered that normal liver contains a carcinostatic factor of unexpected potency,

which is active against homologous tumor cells *in vitro*.

## Experimental Methods

Preliminary experiments were conducted to determine the most suitable experimental conditions, which are simple and dependable, paying special attention to the concentration and amount of tissue extracts in relation to the amount of tumor cells, to the duration of incubating the mixture and to the method of determining the effect on tumor cells. The experimental procedure finally adopted as the standard method was as follows:

Believing that perhaps tumor cells in isolated state may be more easily affected than solid tumor tissue we used the ascites form of Ehrlich carcinoma cells in our tests. Tumor ascites was aspirated 7 to 10 days after intraperitoneal injections and was divided into 2 cc portions in sterilized centrifuge tubes. Tumor cells in each tube was washed once with saline solution and sedimented.

Normal tissue extracts were prepared, unless otherwise stated, always as follows: 1 g of freshly removed tissue was homogenized with 10 cc of physiological salt solution by means of a Potter-Elvehjem homogenizer and rapidly centrifuged at 3,000 rpm for 5 minutes. The turbid supernatant was used as the tissue extract.

One of the various tissue extracts was added in 2 cc amount to the tumor cell sediment above described, the mixture well stirred to make the cell suspension even, and incubated at 37°C for 1 hour, shaking the mixture once or twice during the time. After the incubation, tumor cells were sedimented, discarding the supernatant, re-suspended in the original volume (2 cc) of normal salt solution, and injected into normal mice subcutaneously at right groin in 0.2 cc doses. Cell counts have been made unnecessary by using the overwhelmingly adequate dose of tumor cell suspension, containing some 20~30 million cells. Subcutaneous implantation in the very large dose was chosen in order to facilitate the early determination of the outcome.

Under these experimental conditions sizable tumors can be definitely recognized in 4 or 5 days after implantation in the cases of positive takes. A group of 5 to 8 mice was used for each test, and the final results were recorded at the end of 3 weeks after tumor implantation by killing all the mice and performing autopsy.

## Results

Unequivocal and clean-cut results were obtained under the above experimental conditions, and repeated experiments consistently yielded identical outcome, which may be summarized in Table 1.

These data showed that normal liver extract alone of all kinds of tissue extracts

Table 1. Effect of various tissue extracts on Ehrlich ascites carcinoma cells *in vitro* as assayed by the tumor growth after implantation into normal mice.

| Source of extract | Tumors in implanted mice |
|---|---|
| Liver (mouse) | None (30 mice) |
| Liver (N-F sarcoma-bearing mouse) | Large tumors in 100% (8 mice) |
| Kidney (mouse) | 〃 (5 mice) |
| Spleen ( 〃 ) | 〃 (5 mice) |
| Skeletal muscle ( 〃 ) | 〃 (5 mice) |
| N-F sarcoma ( 〃 ) | 〃 (5 mice) |
| Liver (rat) | None (5 mice) |
| Liver (Hepatoma-bearing rat) | Large tumor in 100% (8 mice) |
| Skeletal muscle (rat) | 〃 (5 mice) |
| Hepatoma ( 〃 )* | 〃 (5 mice) |
| Controls (Incubated in salt solution) | Large tumors in 100% (20 mice) |

* Ascites hepatoma, strain AH-66F of the Sasaki Laboratory. 2.5 g (wet weight) of tumor cells was collected from tumor ascites by centrifugation and homogenized in 5 cc of distilled water, and centrifuged supernatant was used, which represented an extract 5 times more concentrated than the standard tissue extract used throughout the present series of experiments.

exhibited carcinostatic action, which was dramatically clean-cut. Liver extracts from tumor-bearing animals were inactive.

We have not made critical studies on the morphological and biochemical changes produced by the oncostatic factor on tumor cells. Desultory examinations of the cells, even after their incubation with liver extract for so long as 3 hours, showed no such obvious changes as swelling or blebbing of the cytoplasm, which are known to rapidly occur in tumor cells exposed to the action of oncolytic heterologous blood serum. Also, there was no evidence of cytolysis within the period of incubation, and erythrocytes contaminating the tumor cells remained without being hemolysed. The mode of action of the carcinostatic liver factor must at the present be said to be unknown.

The demonstration of the carcinostatic liver factor opened up a vast field for further studies, some of which are already underway in this laboratory. For the present, we limit ourselves to recording a few results which may be of sufficient interest to be mentioned in passing.

**Interaction temperature.** The carcinostatic liver factor reacts with Ehrlich cells *in vitro* even at a low temperature. This fact became apparent when a standard extract-cell mixture was kept at the temperature of 5°C for 24 hours and cells implanted into normal mice in the usual dose. No tumor developed in any of the

5 mice at the site of implantation, while control cells kept in normal salt solution at the same temperature for the same length of time produced large tumors at the site of implantation in the same individul mice.

**Incubation time.** Although we adopted the incubation of extract-cell mixture at 37°C for 1 hour in the standard method, we did obtain in one test 100% negative tumor takes after only 30 minutes' incubation. The interaction between tumor cells and carcinostatic factor may thus be a relatively rapid one, suggesting the possibility that by reducing the number of the tumor cells in the test system the reaction may be shown to complete itself in several minutes.

**Question of bile.** Since bile is the specific product of liver, its possible role in the carcinostatic effect of liver extract cannot be ignored. The difficulty in obtaining mouse bile in sufficient amount for experimental purpose led us to use bovine bile.

25 mg of dried whole bovine bile was dissolved in 10 cc of normal salt solution and 2 cc of it was mixed with 2 cc ascites equivalent of tumor cells and incubated at 37°C for 1 hour, after which the cells were resuspended in 2 cc of salt solution and injected into 5 normal mice in 0.2 cc dose. All the mice rapidly developed tumors.

When the concentration of bile was increased to 50 mg per 10 cc of salt solution and experiment conducted as above, 4 out of 5 implanted mice developed tumors.

It will be seen that in this last experiment the amount of bile contained in the solution is 10 mg per 2 cc. The total solids in 2 cc of the liver extracts of tested and proved potency was found to be around 120 mg including NaCl. If the bile were to account for the carcinostatic action of the liver extract, the bile content of the latter must amount to much over 1/12 of the total solids of the extract.

**Thermostability of the factor.** It is possible to state that the carcinostatic liver factor is highly thermostable and it suffers little loss of its activity after being autoclaved at 120°C under 15 lb pressure for 10 minutes.

The clear solution, only slightly tinged with yellowish brown, obtained by autoclaving liver extract and centrifuging off the coagula, was found to be as active as the original fresh extract, that is, 2 cc of it mixed with 2 cc ascites equivalent of tumor cells and incubated at 37°C for 1 hour completely robbed the cells of tumor producing ability as found when implanted into susceptible mice.

Fractionation of liver extract freed of heat-coagulable matter is in progress.

## Discussion

It has long been known that blood sera of certain animal species show lytic action on heterologous tumor cells when mixed in test tubes. For instance, tested against mouse tumor cells, human serum is highly cytolytic. This heterologous system of oncolytic reaction *in vitro* has recently been demonstrated to consist of a natural

antibody and complement by Kuru *et al.* (3) and by Landy *et al.* (4). It is as a whole a thermolabile system which is inactivated at 55-60°C in 30 minutes.

The carcinostatic liver factor demonstrated in the present study is totally unrelated to this well known heterologous system.

The fact that the liver extract of the rat is apparently as potent as that of the mouse indicates that there is no species specificity in the carcinostatic liver factor, which suggests that extracts from other animal livers may be equally active against not only mouse tumor cells but also tumor cells of other animals, including those of man. In the use of heterologous liver (Herbut and Kraemer (2)) in experiments of this type, however, due precaution must be taken to exclude the complications resulting from the co-existing serum antibody-complement system, which rapidly cytolyses certain heterologous tumor cells. The rat serum has no such serum system which acts upon mouse tumor cells.

One point which may be mentioned in connection with rat liver is the fact that while normal liver yields the carcinostatic factor, the same cannot be obtained from hepatoma cells, apparently showing that the factor becomes deleted when liver cells become cancerized. This, however, is perhaps to be expected *a priori*.

The classical postulate of Freund and Kaminer (5) that normal human serum but not serum of cancer patient brings about lysis of human cancer cells has since fallen into poor repute, because of the great individual variation of the activity among the sera. It should be pointed out, however, that the source of the anti-cancer cell factor in normal blood serum was entirely unknown at that time. If, as we are now strongly inclined to believe, the factor is produced in the liver and is liberated into the circulation, variation in the concentration of the factor in blood sera is only to be expected. It is also probable that the capacity of the liver to produce the oncostatic factor may become greatly reduced in cancer-bearing hosts, since we know that the general liver dysfunction is a common phenomenon in malignant diseases. This idea is supported by our experiments in which livers from tumor-bearing animals failed to yield active extract.

The important problem is that of the homologous carcinostatic system, which was first studied by Freund and Kaminer in blood serum of man and which may be closely related to the homologous carcinostatic liver factor demonstrated by us. Our working hypothesis that this latter may be operative in preventing metastasis formation can be proved only by direct experimental tests.

## Summary

By testing effect *in vitro* of various homologous tissue extracts under accurately defined experimental conditions it was demonstrated that normal liver contains a potent carcinostatic factor, which other tissues failed to yield. The complete failure

of the tumor cells to take, when implanted into susceptible host, was brought about by allowing only 2cc of centrifuged supernatant of mouse liver homogenate, prepared at the rate of 1g of liver in 10cc of normal salt solution, to interact with 2cc ascites equivalent of Ehrlich carcinoma cells at 37°C for 1 hour.

The distinction of this liver factor from the well known oncolytic system of certain heterologous blood sera was pointed out and the possibility of the hepatogenic carcinostatic factor playing a deciding role in the disposal of isolated cancer cells in body fluid and consequently in preventing the formation of metastasis was considered. The importance of homologous carcinostatic system, contrasted to the heterologous one hitherto studied, was especially emphasized.

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[GANN, 52, 203~212; June, 1961]

# MICROSPECTROPHOTOMETRIC MEASUREMENTS OF THE DEOXYRIBONUCLEIC ACID (DNA) CONTENT IN TWO CHROMOSOMAL LINES OF THE EHRLICH ASCITES CARCINOMA[1),2)]

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Microspectrophotometric measurements of the deoxyribonucleic acid (DNA) in individual nuclei have been undertaken in a variety of materials by the use of different methods, as follows: (1) the measurements with ultraviolet light having an absorption peak at 260 mμ (Caspersson 1936, Walker 1958, and some others), (2) those with a single wave length (Caspersson 1950, Swift 1950a, Pollister 1952, Naora 1955, and some others), and (3) those with two wave lengths (Patau 1952, Mendelsohn 1958, Utsumi 1959).

The two-wave-length method served to a great extent for the measurements of the DNA-content of nuclei showing a heterogeneous distribution of Feulgen-dye as compared with other methods which require spherical nuclei with homogeneous distribution of the dye. Further the two-wave-length method is advantageous for measurements of a number of nuclei in actively dividing tumor cells widely different in the DNA-content from cell to cell, and also in irregular-shaped cells at mitosis.

Occurrence of a wide range of chromosome numbers has been reported in tumor cells of animal and human neoplasms by several modern cytologists. The chromosomal situation has generally been understood by studying dividing cells. On the other hand, the results of DNA measurements are helpful for a critical estimation of the ploidy in resting cells.

Data will be presented in this paper which show how accurate a correspondence could be obtained between the DNA-content and the chromosome-number distributions by means of a recently devised microspectrophotometer which was designed by Naora (1955) to be free from Schwarzschild-Villiger effects, adopting the two-wave-length method according to Patau (1952), using two lines of the Ehrlich ascites carcinoma.

The auther wishes to express his sincere thanks to Dr. T.H. Yosida, National Institute of Genetics, Misima, under whose accomodation this work has been carried out in his laboratory. Thanks are offered also to Professor Sajiro Makino for his keen interest in the subject and improvement of this manuscript.

## Material and Methods

A hyperdiploid line of the Ehrlich ascites carcinoma (2N-EL) was brought by Dr. T.H. Yosida from the Children's Cancer Research Foundation, Boston, in 1958 to the National Institute of Genetics, Misima. The line has been maintained from then on by serial transfers into Swiss albino mice. The sampling was made at the 33rd

1) Contributions from the National Institute of Genetics, Misima, Japan, No. 360.

2) Supported by a grant from the Rockefeller Foundation (RF 57178) (Dr. H. Kihara).

generation in Dr. Yosida's laboratory. A hypotetraploid line of the Ehrlich ascites carcinoma (4N-EL) was supplied by the Zoological Institute, Tokyo University. The sampling was made at the 68th generation. The ascites exudates were drawn with a glass capillary from the peritoneal cavity on the seventh day after transfer when the tumors were in sufficient growth suitable for obtaining mitotic figures. Use was made also of monocytes and splenic cells of normal mice as controls, monocytes being obtained from the ascites fluid of Swiss albinos with a glass capillary. The same animals supplied also spleens. They were minced to isolate nuclei.

**Slides for DNA measurements:** A part of the specimens was used for chromosome observations and the other for DNA measurements. The preparations for DNA measurements were made as follows: an aliquot of cell suspension containing ascites carcinoma cells or monocytes or isolated splenic cells from normal animals was smeared on a thin cover-glass (0.13-0.17 mm thick), air-dried, and fixed with freshly prepared Carnoy's solution (absolute alcohol : glacial acetic acid = 3 : 1) for 20 minutes. It was then washed thoroughly with 95% alcohol and stored in 80% alcohol till required. All slides were treated simultaneously by the following procedures: They were washed in distilled water, and hydrolysed with 1N-HCl carefully maintained at 60±0.5°C for ten minutes. Just after hydrolysis the slides were washed in cold water, and stained for 1.5 hours with Schiff's reagent at room temperature. After staining, they were bleached with glycine-buffered $SO_2$-water according to the method of Shibatani and Naora (1952), three times each. After washing with distilled water, they were dehydrated rapidly in alcohol series up to xylol, then mounted with synthetic resin (Bioleit), and covered with a thin cover-glass, similar in thickness to the slides.

**Determination of the duration of hydrolysis:** To determine the optimum duration of hydrolysis important for Feulgen reaction, smear preparations of 4N-EL were hydrolysed: hydrolysis with hydrochloric acid, maintained carefully at 60±0.5°C, was made at intervals of two minutes for a period of time ranging from 4 to 16 minutes. Mean values of Feulgen dye-contents were measured by microspectrophotometry based on 50 nuclei out of each preparation at respective durations of hydrolysis.

**Microspectrophotometer and measurements of DNA:** For cytophotometry, an Olympus microspectrophotometer was employed. For determination of wave lengths, a nucleus which was moderately stained with Feulgen reaction and had an apparently homogeneous distribution ofdye, was taken as the test object. Measuring the transmission at each wave length, an absorption curve for Feulgen-dye was obtained by means of the microspectrophotometer. Based on this curve a couple of wave lengths giving an absorbance of 1 : 2 was chosen, and two wave lengths at 561 mμ and 482 mμ were evaluated.

Computation of the DNA-content in arbitrary units was carried out after Patau's

formula (Patau 1952).

**Measurements of nuclear size:** The diameter of each nucleus at interphase or prophase used for DNA measurements was calibrated by means of an ocular screw-micrometer. Based on the diameter obtained the area of each nucleus was computed.

**Chromosome numbers:** Chromosome counts were made in squash preparations. An aliquot of tumor ascites was placed on a slide, pretreated with a nearly equal volume of deionized water for 10 minutes, stained for 5 minutes with acetic dahlia, and squashed under a coverslip with a finger. Only clear metaphase plates were used for the determination of the chromosome number.

## Results

**Duration of hydrolysis:** In order to determine the optimum duration of hydrolysis, fifty nuclei out of different hydrolysis duration classes were measured by micro-spectrophotometry. The results are shown graphically in Figure 1. It was found that 10 minutes' hydrolysis was the optimum duration for maximum Feulgen reaction.

**DNA-content:** Based on Feulgen preparations the amounts of DNA were measured by microspetrophotometry adopting the two-wave-length method. The results obtained were analysed in relation to the change of the DNA-content during mitosis, and compared with the DNA-content obtained in normal somatic cells of mice such as monocytes and splenic cells. The measurement data were based on one hundred nuclei derived from monocytes, splenic cells and 2N-EL at interphase, 200 nuclei of 4N-EL at interphase, and 50 nuclei from both tumor lines at mitotic phases.

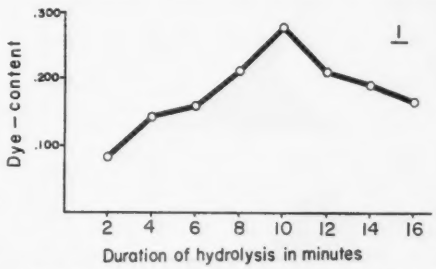
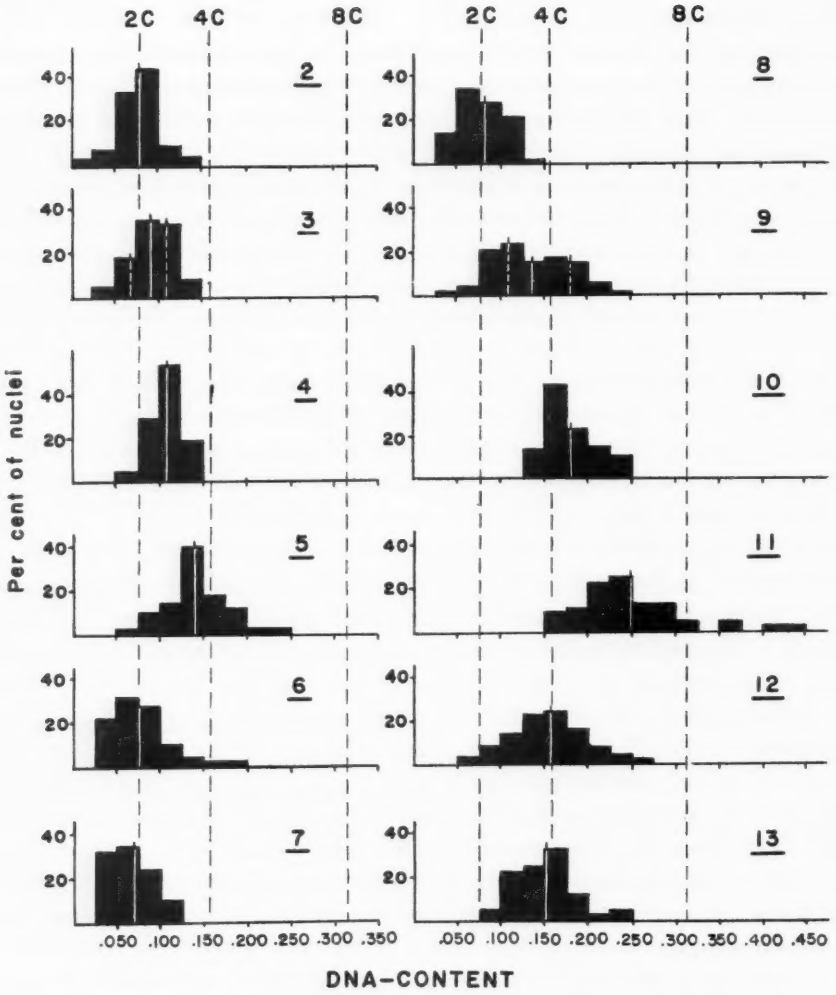



Fig. 1. Feulgen-hydrolysis curve after Carnoy's fixation (each point: mean dye-content of 50 interphase nuclei of 4N-EL).

A diploid value (2C) was calculated from mean values of monocytes and splenic cells. Then, 2C×2 and 2C×4 values were taken as a standard tetraploid (4C) and octoploid (8C) values (dashed lines in Figs. 2 to 13). As shown in Figure 9, the histogram from 4N-EL interphase nuclei is of a bimodal nature. This seems to imply that there occurs a doubling of DNA in interphase nuclei. A unimodal distribution of DNA was obtained in 2N-EL interphase nuclei. It is evident from the above findings that the 4N-EL line is characterized by a bimodal distribution of DNA, while the 2N-EL line by a unimodal one. The distributions of DNA in the 2N-EL and the 4N-EL interphase nuclei were separated into two groups on the basis of the amount of DNA: nuclei containing a large amount of DNA (large amount-nuclei)




Figs. 2 to 13. Frequency distributions of the DNA-content per nucleus. Fig. 2: monocytes. Fig. 3: interphase of 2N-EL. Fig. 4: prophase of 2N-EL. Fig. 5: metaphase of 2N-EL. Fig. 6: anaphase of 2N-EL. Fig. 7: telophase of 2N-EL. Fig. 8: splenic cells. Fig. 9: interphase of 4N-EL. Fig. 10: prophase of 4N-EL. Fig. 11: metaphase of 4N-EL. Fig. 12: anaphase of 4N-EL. Fig. 13: telophase of 4N-EL.

and those containing a small amount of DNA (small amount-nuclei). The mean amounts of DNA in large amount- and small amount-nuclei are shown in Figures 3 and 9 with dashed lines. The DNA-content of small amount-nuclei at interphase was found to be identical with that of anaphase and telophase nuclei, while the DNA-

content of large amount-nuclei at interphase was nearly similar to that of prophase nuclei. The amounts of DNA at metaphase nuclei were just twice as much as those of anaphase and telophase nuclei. The amounts of DNA of prophase nuclei corresponded to approximately three-quarters of metaphase nuclei.

Changes in the course of mitosis of the DNA-content of the 2N-EL line were found to be parallel to those of 4N-EL line. The DNA-content of both lines increased in prophase and metaphase nuclei and then decreased down to half value at anaphase and telophase (Table 1).

Table 1. Mean value of the DNA-content at respective mitotic phases in hyperdiploid and hypotetraploid lines of the Ehrlich ascites carcinoma and in normal somatic cells of mice.

| | Interphase | | | Prophase | Metaphase | Anaphase | Telophase |
|---|---|---|---|---|---|---|---|
| | | s.a.n.* | l.a.n.** | | | | |
| 2N-EL | 0.094 | 0.069 | 0.111 | 0.109 | 0.141 | 0.077 | 0.070 |
| 4N-EL | 0.136 | 0.109 | 0.180 | 0.180 | 0.247 | 0.156 | 0.149 |

Monocytes 0.077 Splenic cells 0.080

* s.a.n. = small amount-nuclei at interphase.

** l.a.n. = large amount-nuclei at interphase.

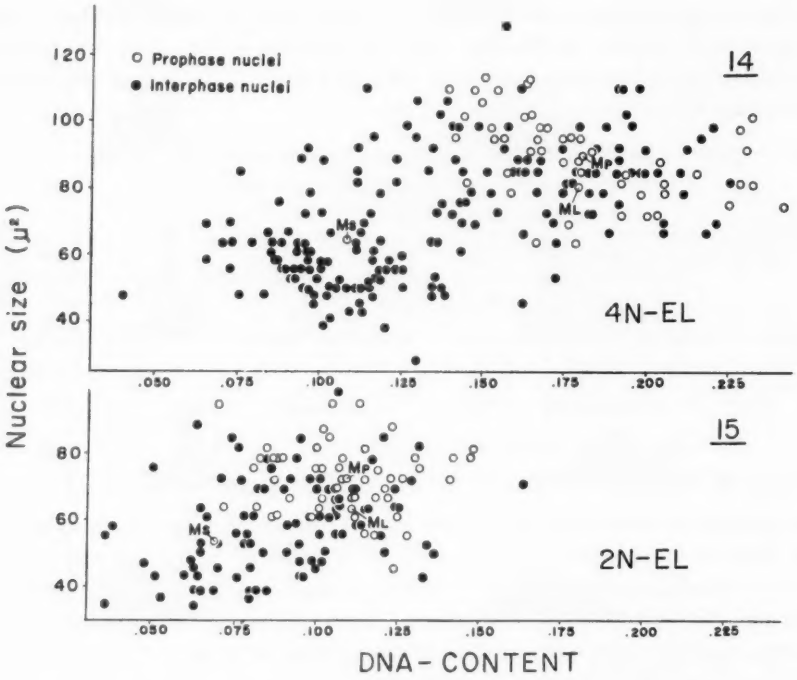
**DNA-content in relation to the nuclear size:** The size of a nucleus was measured on the basis of its diameter. Since the nuclei in smeared and dried preparations assumed a discoidal shape with little difference of thickness, the area obtained based on the diameter may be of relative nature. The correlations of the DNA-content to the nuclear size in each nucleus were shown in Figures 14 and 15. It was shown that there was a correlation between nuclear size and the DNA-content, and that the histogram of nuclear size showed a bimodal distribution with a similar feature to the DNA-content, especially in 4N-EL interphase nuclei. The variation of nuclear size in prophase nuclei that is correlated to the DNA-content was found nearly to

Table 2. Mean value of the DNA-content and nuclear size in interphase and prophase nuclei in two lines of the Ehrlich ascites carcinoma.

| | No. of nuclei measured | | Mean value of DNA-content* | | Mean value of nuclear size** | |
|---|---|---|---|---|---|---|
| | 2N-EL | 4N-EL | 2N-EL | 4N-EL | 2N-EL | 4N-EL |
| Small amount-nuclei at interphase | 41 | 124 | 0.069 | 0.109 | 53.795 | 64.635 |
| Large amount-nuclei at interphase | 59 | 76 | 0.111 | 0.180 | 63.256 | 85.100 |
| Prophase nuclei | 50 | 50 | 0.109 | 0.180 | 72.688 | 89.866 |

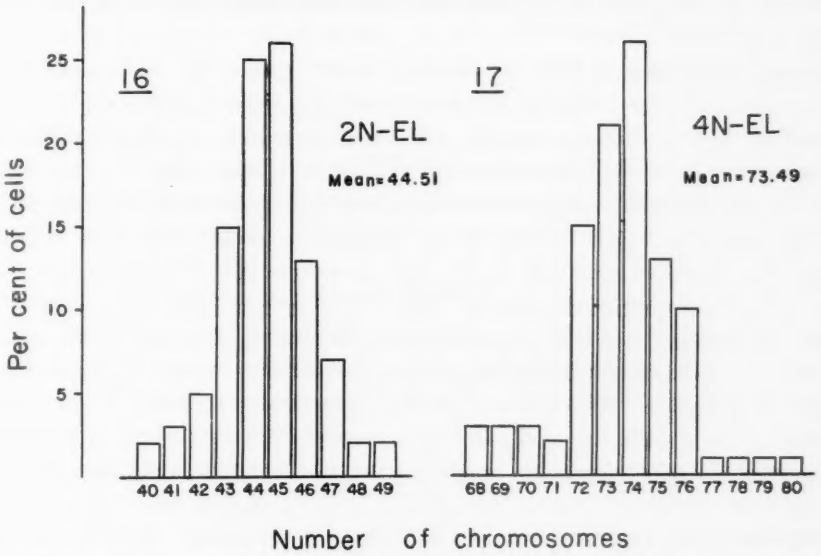
* In arbitrary unit ** In square microns.

correspond to the variation of large amount-nuclei at interphase (empty circles in Figures 14 and 15). The rate of increment of nuclear size in each tumor line was less than that of the DNA-content (Table 2).




Figs. 14 and 15. The relation between nuclear size and the DNA-content in hypotetraploid Ehrlich ascites carcinoma cells (Fig. 14) and hyperdiploid Ehrlich ascites carcinoma cells (Fig. 15) at prophase and interphase. Ms: mean of small amount-nuclei at interphase. ML: mean of large amount-nuclei at interphase. Mp: mean of prophase nuclei.

**Distribution of the chromosome-number:** Chromosome counts were made in two lines of Ehrlich ascites carcinoma based on clear metaphase plates obtained from squash preparations. One hundred metaphases were observed in each line. It was found that the modal chromosome-number was 45 for 2N-EL with a variation from 40 to 49, and 74 for 4N-EL showing a fluctuation between 68 and 80 (Figs. 16 and 17). In both lines, 85% of cells showed the chromosome-distributions within a narrow range, with the mean values at 44.51 for 2N-EL and 73.49 for 4N-EL. The ratio of mean value of 2N-EL to 4N-EL was 1 : 1.67, while the modal number was at a ratio of 1 : 1.64.




Figs. 16 and 17. Distribution of chromosome numbers in hyperdiploid Ehrlich ascites carcinoma (Fig. 16) and hypotetraploid Ehrlich ascites carcinoma (Fig. 17).

Table 3. Mean value of the chromosome number, measured and expected values of the DNA-content, and ratios.

| | Chromosome-number | | | DNA-content | | | |
|---|---|---|---|---|---|---|---|
| | No. of cells observed | Mean | Ratio* | No. of cells measured | Mean*** | Ratio* | Expected value*** |
| Monocytes | — | 40 | 1.000** | 100 | 0.077 | 1.000** | 0.079** |
| Splenic cells | — | 40 | | 100 | 0.080 | | |
| 2N-EL | 100 | 44.51 | 1.113 | 100 | 0.094 | 1.185 | 0.087 |
| 4N-EL | 100 | 73.49 | 1.837 | 200 | 0.136 | 1.732 | 0.144 |

* Ratio of mean values of the chromosome number and DNA-content in two lines of the Ehrlich ascites carcinoma to those in normal somatic cells.

** From average values of monocytes and splenic cells.

*** In arbitrary unit.

## Discussion

**Duration of hydrolysis:** The optimun duration of hydrolysis for maximum Feulgen reaction varies according to the fixatives and tissues employed. Working with cartilaginous cells of *Rana pipiens* and bull sperm nuclei fixed with Carnoy's solution, Di Stefano (1948) and Leuchtenberger (1958) found that 10 minutes hydrolysis gave rise to a maximum amount of Feulgen-dye. The most suitable duration of hydrolysis

of the Ehrlich ascites carcinoma obtained in this study closely agree with the result of those authors.

**Bimodal distribution of DNA in interphase nuclei:** As shown in Figures 9 and 14, the distribution of the DNA-content of nuclei was found to be bimodal in 4N-EL interphase nuclei. A similar situation was found to occur in the tetraploid Ehrlich ascites carcinoma by Richards (1955), and Utsumi and Seno (1960). Kasten (1960) and Freed and Hungerford (1959) reported a bimodal distribution of DNA in interphase nuclei of the hyperdiploid Ehrlich ascites carcinoma. Deeley *et al.* (1954) had also observed in nuclei of chick and mouse heart in culture a bimodal distribution of interphase nuclei: one of the bimodal distributions corresponded to the DNA-distribution of anaphase and telophase nuclei, and the other to that of prophase nuclei. On the other hand, the diploid Ehrlich ascites carcinoma showed the unimodal feature in DNA-distribution. This finding might be interpreted as a result of close overlapping of two modes. In other words, the bimodal DNA-distribution in the tetraploid Ehrlich ascites carcinoma might result from an increase of chromosomal ploidy.

The above features accord with the conclusion of Swift (1950), Howard and Pelc (1952), Kimball and Barka (1959) that DNA synthesis occurs during interphase, in disagreement with the argument of Lison and Pasteels (1951) that the synthesis of DNA takes place during telophase.

**Nuclear size in relation to the DNA-content:** It has been shown in the present study that the DNA-content of nuclei is correlated to the size of interphase and prophase nuclei. But Swift (1950) showed no correlation between them. The disagreement may depend upon the cellular activity, since tumor cells are growing with higher activity in comparison with normal somatic cells. The distribution of DNA in interphase nuclei of 4N-EL was wider than that of 2N-EL. A similar observation was made by Alfert *et al.* (1955) and Frazer and Davidson (1953) in rat liver nuclei.

It was observed in the two lines of the Ehrlich ascites carcinoma that the size of prophase nuclei was slightly larger in mean value than that of large amount-nuclei at interphase, while the DNA-content was nearly identical in the two. It seems probable that the growth in size of nuclei may be controlled by cytoplasmic elements other than DNA. In this connection the results of Prescott (1960) are of most interest: he showed working with *Tetrahymena* that the synthesis of DNA is completed prior to the completion of protein synthesis.

**Chromosome numbers and the DNA-content:** It was shown in the present study that the mean chromosome number of the Ehrlich carcinoma is virtually proportional to the mean value of the DNA-content in interphase nuclei. There were found no significant difference between measured values and expected ones of the DNA-content

($X^2=0.00105$, $0.95<P<0.98$) (Table 3). The latter value was computed from the mean value of the chromosome number. A similar feature was found by Freed and Hungerford (1957) and Alfert and Swift (1953) to occur in hyperdiploid (S=45–46) Lettré-Ehrlich ascites carcinoma and rat tissue cells, respectively: they showed that the DNA-content of nuclei was constant and proportional to the number of chromosomes. On the other hand, Kasten (1960) found that the DNA-content of hyperdiploid Ehrlich ascites carcinoma (modal chromosome number: 46) was consistent with that of liver cells (40 chromosomes) of mice.

## Summary

The DNA-content of hyperdiploid and hypotetraploid tumor cells of the Ehrlich ascites carcinoma was measured by Feulgen-microspectrophotometry, adopting the two-wave-length method (482 mμ and 561 mμ) in comparison with that of normal mouse monocytes and splenic cells.

It was found that the DNA-content of nuclei was proportional to the number of chromosomes. The change of the DNA-content during mitosis was measured: it was shown that the mean amounts of DNA of small amount-nuclei at interphase were identical with those of anaphase and telophase nuclei, being just half the metaphasic amount. The amounts of DNA of prophase nuclei correspond to approximately three-quarters of those of metaphase nuclei. Based on the results of measurements of the DNA-content in relation to size of nuclei, the mechanism of DNA synthesis was discussed in connection with cell growth during interphase and prophase. A similar pattern of the correlation between nuclear size and the DNA-content was found to occur in hyperdiploid and hypotetraploid lines.

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